

Environment-microbe-host interactions: understanding the relationship between the external environment, gut microbiome diversity, and host immunocompetence

By

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General Abstract

Microorganisms inhabiting the gastrointestinal tract of vertebrates co-exist with their hosts and may provide them with health and physiological benefits. However, environment-microbe-host interactions within gut microbiome communities (GMCs) are poorly understood. In this study deer mice (*Peromyscus maniculatus*) were used to determine a) how the natural (wild) environment influences GMCs compared to captivity and b) how microbes associated with the natural environment as well as overall GMC α -diversity influences host immunocompetence. Captive- and wild-born deer mice were sampled twice (initial and post-translocation phases), two-weeks apart. After the initial sampling period a sub-sample of deer mice were reciprocally translocated between environments to assess how the external environment influences host GMCs and immunocompetence. Non-translocated individuals served as captive- and wild-born controls. GMCs were analysed via fecal samples, sequenced using 16S rRNA next –generation Illumina HiSeq sequencing. Host immunocompetence was determined using blood cell counts and a functional immune challenge (i.e. bacteria killing assay). Captive individuals possessed less diverse GMCs compared to wild individuals. Individuals translocated from captivity to the wild increase in GMC α -diversity, while individuals reciprocally translocated experienced a decrease. Individual's GMCs clustered closer together with deer mice sharing the same environment. In natural environments deer mice had higher abundances of *Ruminococcaceae*, *Helicobacteraceae*, and *Lachnospiraceae* spp., than in captivity. No strong correlations were found between GMC α -diversity and host immunocompetence. Findings suggest that despite containing less diverse GMCs in captivity, upon reintroduction to natural environments deer mice GMCs rapidly changed, homogenizing with deer mice in the same environment. Future experiments should further study the effects of reduced exposure to *Ruminococcaceae*, *Helicobacteraceae*, and *Lachnospiraceae* during early-life stages, to better understand environment-microbe-host interactions.

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Table of Contents

General Abstract.....	iii
Acknowledgements	iv
List of Tables	vii
List of Figures.....	viii
General Introduction	1
<i>Literature cited.....</i>	<i>15</i>
Chapter 1: Environmental influences on α- and β-diversity of gut microbiome communities in deer mice (<i>Peromyscus maniculatus</i>).....	20
1.1 Abstract.....	21
1.2 Introduction.....	22
1.3 Methods.....	25
1.4 Results	34
1.5 Discussion.....	43
1.6 Literature cited.....	56
Chapter 2: Relationships between gut microbiome diversity and host immunocompetence	92
2.1 Abstract.....	93
2.2 Introduction.....	94
2.3 Methods.....	97
2.4 Results	103
2.5 Discussion.....	104
2.6 Literature cited.....	109
General Discussion.....	116
<i>Literature cited.....</i>	<i>123</i>

List of Tables

Chapter 1

Supplementary Table 1-1: Mean relative abundance of microbes at different taxonomic classification levels. Non-parametric unpaired Mann-Whitney U tests were performed to test for significant differences between deer mice born in captivity versus individuals the wild. Significant differences were seen for a number of microbes at different taxonomic levels. 79

Supplementary Table 1-2: List containing the mean relative abundance of bacteria at the phylum, class, order and family level, during the post-translocation sampling period for deer mice in all four experimental groups. 80

List of Figures

1 Chapter 1

Figure 1-1: Diagram demonstrating the experimental design followed for this study. Pregnant dams were captured in the wild (May-early June) and transferred to the Algonquin Provincial Park Wildlife Research Station so that their offspring would be born in captivity. Three-weeks later after the captive juveniles were born their initial fecal samples were collected. A sub-sample of captive born individuals were then translocated to the wild (Captive-Wild group). Mice that remained in captivity (Captive-Captive group) along with their translocated siblings were then recaptured and sampled again two-weeks (post-translocation sampling period) after the initial sampling period had occurred. Wild juvenile deer mice were sampled in the wild during the initial sampling period, a sub-sample of mice were then translocated to captivity (Wild-Captive group). Two-weeks later mice translocated from the wild to captivity as well as mice that remained in the wild (Wild-Wild group) were sampled again (post-translocation sampling period). 63

Figure 1-2: Initial capture results show that there were differences in α -diversity levels among the four experimental groups during the initial sampling phase of the experiment. Chao1 ($F_{1,3} = 32.23$, $p < 0.01$) and Fisher α -diversity ($F_{1,3} = 28.07$, $p < 0.01$) indices show significant differences between deer mice born in different environments ($p < 0.01$; for all comparisons among groups where individuals were born in different environments). Shannon's diversity index ($\chi_{1,3} = 19.08$, $p < 0.01$) showed similar results as Chao1 and Fisher except there was no significant difference between the Captive-Wild/Wild-Captive groups ($p = 0.11$). **** indicates $p < 0.0001$, *** indicates $p < 0.001$ and ** indicates $p < 0.01$ 64

Figure 1-3: Chao1, Fisher and Shannon α -diversity levels displayed marginal or significant difference between experimental groups during the post-translocation sampling period. There was a significant difference between the experimental groups during the post-translocation sampling period when using the Chao1 α -diversity index ($F_{1,3} = 4.08$, $p < 0.05$). Wild-Wild deer mice had significantly more diverse gut microbiome communities compared to Captive-Captive deer mice ($p = 0.01$), with a marginal difference between Wild-Wild/Wild-Captive deer mice ($p = 0.069$). The Fisher α -diversity scores ($F_{1,3} = 5.074$, $p < 0.01$) showed significant differences between Wild-Wild/Wild-Captive deer mice ($p < 0.05$) and Wild-Wild/Captive-Captive deer mice ($p < 0.01$). Shannon's index scores ($\chi_{1,3} = 10.44$, $p < 0.05$) showed a significant difference between Wild-Wild/Wild-Captive deer mice ($p < 0.05$) and a marginal difference between the Wild-Wild/Captive-Captive groups ($p = 0.055$). ** indicates $p < 0.05$ and * indicates $p < 0.1$ 65

Figure 1-4: Venn diagram displaying all the OTUs identified in deer mice during the post-translocation phase of the experiment. Wild-Wild individuals contained 519 (21.3%) unique OTUs, more than three times the amount of unique OTUs belonging to any of the other experimental groups. A large number of OTUs (i.e. 774 OTUs [31.8%]) were shared between all four experimental groups. 66

Figure 1-5: Changes in α -diversity levels between initial and post-translocation sampling periods were identified within different groups using different α -diversity indices. Chao1 α -diversity levels showed a significant increase for deer mice in the Captive-Wild group ($p < 0.01$) as well as a significant decrease for deer mice in the reciprocally translocated Wild-Captive group ($p < 0.01$). The only significant change in α -diversity between sampling periods seen when using Fisher's ($p < 0.01$) and Shannon's ($p = 0.08$) indices was a decrease for deer mice in the Wild-Captive group. ** indicates $p < 0.05$ and * indicates $p < 0.10$ 67

Figure 1-6: Principle coordinate analysis (PCoA) ordinations using weighted UniFrac measurements determined that the gut microbiome communities of deer mice born in the same environment clustered together, during the initial sampling period (adonis: $F_{1,3} = 7.20$, $R^2 = 0.25$, $p < 0.01$). 68

Figure 1-7: The relative abundance of microbes (classified at the phylum level) that compose the gut microbiome communities (GMCs) of deer mice born in the captive and wild environments. The GMCs of deer mice born in captive environments are dominated by Bacteroidetes followed by Firmicutes. The GMCs of deer mice born in wild (natural) environments are dominated by Firmicutes followed by Bacteroidetes, the opposite of captive born deer mice. Proteobacteria also appear to be more consistently abundant in wild born deer mice compared to captive born individuals. Each bar represents the GMC of an individual deer mouse. 69

Figure 1-8: Side-by-side comparison of the mean relative abundance of families which made up at least $>1\%$ of the total gut microbiome community, within at least one individual, during the initial capture period for deer mice born in captivity and the wild. Significant differences occur between the *Ruminococcaceae* ($p < 0.001$), *Lachnospiraceae* ($p < 0.01$), *Helicobacteraceae* ($p < 0.01$), *S24-7* ($p < 0.001$) and *Enterobacteriaceae* ($p < 0.05$)†. *Lactobacillus* showed no significant difference between captive and wild groups during the initial sampling period. †This relationship seemed to be driven by just two individuals, see Supplementary Figure 1-3. 70

Figure 1-9: Linear discriminant analysis (LDA) effect size (LEfSe) determined that there were a number of microbial families that were more abundant in the GMCs of wild deer mice compared to GMCs of captive raised deer mice (initial sampling period), including *Ruminococcaceae*, *Helicobacteraceae*, and *Lachnospiraceae*, which composed $>1\%$ of gut microbiome communities in deer mice. Alternatively, microbial families *S24-7* and *Enterobacteriaceae* (composed $>1\%$ of gut microbiome communities in some captive deer mice individuals) were found to be significantly more abundant in captive deer mice. 71

Figure 1-10: Constrained Analysis of Principle Coordinates (CAP) ordination method was used to visualize differences between captive and wild deer mice gut microbiome communities (GMCs) (A), during the initial sampling period. The GMCs of deer mice from the initial capture period, grouped together depending on where they were born (captivity or in the wild). The shaded section

of figure A is represented in figure B, demonstrating which OTUs were the most influential in separating captive and wild enterotypes. Below is a heat map showing the z-scores (indicates above or below average abundance) for the top ten most influential operation taxonomic units (OTUs) for both captive and wild enterotypes. 72

Figure 1-11: Phylogenetic tree containing the top ten most influential Operational Taxonomic Units (OTUs) for captive and wild enterotypes during initial and post-translocation sampling periods. Black squares indicate that an OTU was characteristic for the captive enterotype during the initial capture stage, while white squares indicate that an OTU was characteristic among gut microbiome communities for deer mice in captivity during the post-translocation sampling period. Black circles indicate that an OTU was important for the wild enterotype during the initial capture stage, while white squares indicate that an OTU was important among gut microbiome communities for deer mice in wild during the post-translocation sampling period. A number of *S24-7* microbes were associated with captivity, while *Helicobacteraceae* and *Ruminococcaceae* microbes were associated with the wild environment. *Lachnospiraceae* microbes did not show any clear clustering. Inter-genus specializations occurred within the *Lactobacillaceae* family..... 73

Figure 1-12: Principle coordinate analysis (PCoA) ordinations using weighted UniFrac measurements determined that the gut microbiome communities of neighbouring deer mice in the same environment post-translocation clustered together (adonis: $F_{1,33} = 3.20$, $R^2 = 0.10$, $p < 0.01$). 74

Figure 1-13: Mean relative abundance of different microbial families, which represented at least >1% or more of the total GMC within at least one individual deer mouse during the post-translocation sampling period. The microbial family *S24-7* was the most dominant in deer mice that were in captivity during the two-week translocation phase of the experiment (Captive-Captive and Wild-Captive groups). Alternatively, *Ruminococcaceae* was the most dominant microbial family in the GMC of deer mice who were in the wild for the translocation phase of the experiment (Wild-Wild and Captive-Wild groups). 75

Figure 1-14: Constrained Analysis of Principle Coordinates (CAP) ordination method was used to visualize differences between captive and wild deer mice gut microbiome communities (GMCs) (A) during the post-translocation sampling period. GMCs of deer mice from the post-translocation sampling period grouped together depending on the environment they were in during the translocation phase of the experiment. The shaded section of figure A is represented in figure B, demonstrating which operational taxonomic units (OTUs) were the most influential in separating captive and wild enterotypes post-translocation. Below is a heatmap showing the z-scores (indicate above or below average abundance) for the top ten most influential OTUs for captive and wild enterotypes during the post-translocation sampling period. 76

Figure 1-15: Linear discriminant analysis (LDA) effect size (LEfSe) determined that microbial families including *Rikenellaceae*, *Odoribacteraceae* and *S24-7* were more abundant during the initial sampling period, compared to the post-translocation sampling period, in deer mice that were

translocated from captivity to the wild (C-W). During the post-translocation sampling period microbial families including *Dehalobacteriaceae*, *Paraprevotellaceae* and *Ruminococcaceae*, as well as the phylum Proteobacteria were significantly more abundant in C-W deer mice GMCs compared to C-W GMC samples acquired during the initial sampling period..... 77

Figure 1-16: Constrained Analysis of Principle Coordinates (CAP) ordination method was used to visualize the difference between captive and wild born deer mice post-translocation. Each color corresponds to a different litter that was born in captivity, while individuals born in the wild are all colored grey since their dams are unknown. There does not appear to be any clear clustering between littermates in either captivity or the wild post-translocation.....78

Supplementary figures

Supplementary Figure 1-1: Principle Coordinate Analysis ordinations using Bray-Curtis dissimilarity measurements demonstrate that A) deer mice born in captive or wild environments cluster together with deer mice born in the same environment (Adonis: $F_{1,64} = 3.80$, $R^2 = 0.15$, $p < 0.001$) and B) post-translocation deer mice group cluster closer with individuals sharing the same environment rather than where they were born (Adonis: $F_{1,33} = 4.81$, $R^2 = 0.13$, $p < 0.001$). 81

Supplementary Figure 1-2: Principle Coordinate Analysis ordinations using unweighted UniFrac measurements show that THE GMCs of deer mice born in the same environment (A) clustered together (adonis: $F_{1,64} = 3.20$, $R^2 = 0.13$, $p < 0.001$), however, during the post-translocation stage (B) the external environment surrounding deer mice determined which individuals clustered closer together based on GMC composition (Adonis: $F_{1,3} = 2.68$, $R^2 = 0.07$, $p < 0.001$). 82

Supplementary Figure 1-3: The abundance of OTUs which were determined to be enterotype-defining during either the pre-translocation or post-translocation sampling periods. All OTUs had a mean relative abundance $\geq 1\%$ in at least one of the four groups during either the pre- or post-translocation sampling period. A number of OTUs are shown to demonstrate a strong relationships with the natural environment, represented by high mean relative abundances within Wild-Captive and Wild-Wild groups during the pre-translocation sampling period and high mean relative abundances in Captive-Wild and Wild-Wild groups post-translocation.....83

Supplementary Figure 1-4: Pairwise analysis of individuals from the Captive-Captive group, examining the change in relative abundance of families which make up at least 1% of the gut microbiome community (in at least one individual). Significant difference was seen in the family *Desulfovibrionaceae* ($p = 0.021$) and marginal differences in *Enterobacteriaceae* ($p = 0.059$)†, *Lachnospiraceae* ($p = 0.059$) and *Lactobacillaceae* ($p = 0.059$). †This marginal decrease seems to be largely driven by only two individuals in the group. ** indicates $p < 0.05$ and * indicates $p < 0.1$ 84

Supplementary Figure 1-5: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be important during the initial or post-translocation sampling periods. Deer mice in the Captive-Captive group demonstrated significant decreases in OTU 1 (*Lactobacillus murinus*, $p = 0.017$), and OTU 37 (*S24-7*, $p = 0.014$). Significant increases were found for OTU 11 (*Clostridiaceae*, $p = 0.01$), OTU 21 (*Lachnospiraceae*, $p = 0.024$), and OTU 302 (*Clostridiaceae*, $p = 0.031$). 85

Supplementary Figure 1-6: Wilcoxon paired analysis was used to examine the change in relative abundance of families which made up at least 1% of the gut microbiome community (in at least one deer mouse) of deer mice from the Captive-Wild group. Marginally significant differences were seen in the microbial families *Helicobacteraceae* ($p = 0.059$), *Rikenellaceae* ($p = 0.059$), and *S24-7* ($p = 0.059$). ** indicates $p < 0.05$ and * indicates $p < 0.1$ 86

Supplementary Figure 1-7: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be characteristic of captive and wild enterotypes during the initial and post-translocation sampling period was conducted to assess significant changes within the gut microbiome communities of deer mice in the Captive-Wild group. Significant increases ($p < 0.05$) were found for OTU 13 (*Ruminococcaceae*), OTU 185 (*Ruminococcaceae*), OTU 256 (*Clostridiaceae*), OTU 302 (*Clostridiaceae*), OTU 36 (*Clostridiaceae*), OTU 6 (*Helicobacter rodentium*), OTU 60 (*Lachnospiraceae*), OTU 622 (*Lachnospiraceae*), OTU 641 (*Lachnospiraceae*), OTU 8 (*Helicobacter aurati*) and OTU 92 (*Lachnospiraceae*), with OTU 51 (*Ruminococcaceae*, $p = 0.06$) also showing a near significant increase. Significant decreases ($p < 0.05$) were seen among OTU 2 (*Lactobacillus intestinales*), OTU 321 (*S24-7*), OTU 37 (*S24-7*), and OTU 9 (*S24-7*)..... 87

Supplementary Figure 1-8: Wilcoxon paired analysis was used to examine the change in relative abundance of families which made up at least 1% of the gut microbiome community (in at least one deer mouse) of deer mice from the Wild-Captive group. Significant difference in were seen in families *Helicobacteraceae* ($p = 0.014$), *Prevotellaceae* ($p = 0.036$), and *Ruminococcaceae* ($p = 0.042$). ** indicates $p < 0.05$ and * indicates $p < 0.1$ 88

Supplementary Figure 1-9: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be characteristic of captive and wild enterotypes during the initial and post-translocation sampling period was conducted to assess significant changes within the gut microbiome communities of deer mice in the Wild-Captive group. Significant increases ($p < 0.01$) was only found for OTU 26 (*Eubacterium*). Significant decreases ($p < 0.01$) were seen among OTU 13 (*Ruminococcaceae*), OTU 185 (*Ruminococcaceae*), OTU 60 (*Lachnospiraceae*), OTU 622 (*Lachnospiraceae*) and OTU 92 (*Lachnospiraceae*) as well as ($p < 0.05$ for the following) OTU 256 (*Clostridiaceae*), OTU 40 (*Rumincococcaeae*) and OTU 641 (*Lachnospiraceae*). 89

Supplementary Figure 1-10: Wilcoxon paired analysis was used to examine the change in relative abundance of families which made up at least 1% of the gut microbiome community (in at least one deer mouse) of deer mice from the Wild-Wild group. Only a marginal difference was seen in *Rikienellaceae*. ** indicates $p < 0.05$ and * indicates $p < 0.1$ 90

Supplementary Figure 1-11: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be characteristic of captive and wild enterotypes during the initial and post-translocation sampling period was conducted to assess significant changes within the gut microbiome communities of deer mice in the Wild-Wild group. No significant changes were seen among any of the top influential OTUs in deer mice in the Wild-Wild group. 91

2 Chapter 2

Figure 2-1: Mann-Whitney U test was used to test for significant differences between bacteria killing ability of individuals born in captivity ($n = 27$) and the wild ($n = 24$). A marginal difference was seen between the two groups ($W = 372, p = 0.053$).....113

Figure 2-2: No significant correlations appear to be present between Chao1, Fisher's, or Shannon's α -diversity indices and bacteria killing ability ($n = 63$).....114

Figure 2-3: A marginally significant correlation ($R^2 = 0.13, p = 0.05$) was seen when comparing the change in Shannon's diversity and bacteria killing ability over the two week translocation period ($n = 17$).....115

3. General Discussion

Figure 3-1: A visual representation of the how this study fills current gaps within the existing literature and how the findings act as a starting point from which future research can start from. Dark grey circles represents the focus of existing research, while the light grey circles represent the focus of this study (research that current does not exist within the current literature). Future directions for research is also outlined.....126

General Introduction

Introduction to the Gut Microbiome

The term ‘microbiome’ refers to microbial communities (often referred to as a host’s microbial genome), that are composed of trillions of Bacteria, Archaea, and Eukaryotes (Martiny et al. 2006). A number of distinctive microbiomes are found on vertebrate hosts throughout the body, including the skin, gut, vagina, and mouth (Round and Mazmanian 2009). Microbiomes not only differ among host species, but also exhibit temporal and spatial differences within individual hosts (Bobbie et al. 2017). Specific species within the microbiome can have a surprisingly large influence on the fitness and health of their host. For example, the bacterium *Vibrio fischeri* provides its host, the marine squid (*Euprymna scolopes*), with the ability to produce light, which allows the squid to camouflage (Hooper 2004). Alternatively, some bacterial species may lead to the dysfunction of microbiome-related functions (e.g. intestinal swelling and inflammation) within their hosts, should their presence within the bacterial community become too prevalent (e.g. *Clostridium difficile* [van d’er Waaij 1989; Moloney et al. 2014]).

Microbial species found within the gastrointestinal tracts of host organisms are collectively referred to as the gut microbiome (Ley et al. 2008b, Mulder et al. 2009, Lee and Mazmanian 2010). The gastrointestinal tract is initially colonized during early life stages by species which develop parasitic, mutualistic, or commensal relationships with their host (Cerf-Bensussan and Gaboriau-Routhiau 2010, Chung and Kasper 2010, Wang et al. 2014, Gavish et al. 2014). Bacteria comprise the majority of these relationships in vertebrates and are the most influential group of organisms within the gut microbiome (Macpherson and Harris 2004, Sonnenburg et al. 2004)

Hosts provide microbial species with an environment that is sheltered, and rich in resources (Smith et al. 2007). Additionally, mammalian hosts also provide microbes with an environment that maintains a stable temperature (Smith et al. 2007). In return microbial species within the gut microbiome are responsible for performing a number of vital functions including, identification of and protection against pathogens (Stecher 2010), metabolic functions (Hooper 2002), brain development (Heijtz 2001), protecting the intestinal epithelium (Falk 1998), and immune system development (Cash and Hooper 2005, Round and Mazmanian 2009). Due to the essential tasks the gut microbiome performs, it is thought to have developed mutualistically with its host's immune system (Hooper et al. 2002, Round and Mazmanian 2009, Cerf-Bensussan and Gaboriau-Routhiau 2010, Nelson et al. 2013, Zhang et al. 2014a), which needs to recognize and tolerate non-pathogenic species, while eliminating harmful pathogenic species (Cash and Hooper 2005, Kelly et al. 2007, Chung and Kasper 2010, Lee and Mazmanian 2010, Cahenzli et al. 2013, Sommer and Bäckhed 2013).

Evolutionary Perspective and Importance of the Gut Microbiome

Acquisition and maintenance of diverse gut microbiomes is suggested to have been favored by selection, by allowing hosts to perform metabolic functions such as energy and nutrient extraction, making it unnecessary for the host to perform these functions on their own (Hooper 2004, Ley et al. 2008a, Cahenzli et al. 2013). By examining the gut microbiome of various vertebrate species it is possible to see distinct microbial communities (enterotypes) resembling evolutionary lineages (Ley et al. 2005, 2008a). For example, regardless of geographic location, mammalian carnivores, herbivores, and omnivores all possess unique enterotypes. Differences can even be seen in more specific groupings such as hind- and foregut fermenters (Ley et al. 2008a).

The composition of the gut microbiome of vertebrates reflects natural selection at both the microbial and host level (Hooper 2004, O'Hara and Shanahan 2006). At the microbial level, species interact to compete for resources, with selection favoring microbial species possessing specific niches or functions (Ley et al. 2008a, Cho and Blaser 2012). Microbial species composition are shaped by two different selection pressures, as they have to draw enough nutrients from their hosts to survive in a highly competitive environment, without harming their host, thus risking being targeted by the hosts immune system (Dillion et al. 2005).

Due to the strong selection pressures host-microbe relationships evolved under, it has been proposed that phylogenetic relatedness among host species should be mirrored in their gut microbiome communities, a process known as phylosymbiosis (Brooks et al. 2016). Using a variety of taxa and species (including *Peromyscus*) Brooks et al. (2016) provided evidence supporting the concept of phylosymbiosis, by demonstrating the presence of unique microbial communities among distantly and closely related species under controlled laboratory conditions.

Brief Introduction to the Architecture and Evolution of the Immune System

The immune system serves as a mechanism by which organisms are able to protect themselves from harmful foreign substances, to maximize fitness (Viney et al. 2005, Abolins et al. 2011). The immune system of vertebrates is composed of two essential cellular systems, the innate immune system and the adaptive immune system.

The innate immune system evolved before the divergence of vertebrates and invertebrates, explaining similarities between insect, and mammalian immune systems (Kimbrell and Beutler 2001). The innate immune system serves as an instantaneous response to a wide range of foreign pathogens (Kimbrell and Beutler 2001) and is characterized by a variety of cells including: resident non-pathogenic bacteria, neutrophils, macrophages, and phagocytes, all of which function to

eliminate invading pathogens (Hofmeyr 2001, Janeway et al. 2001, Demas et al. 2011). As some pathogens are able to evade the innate immune system, the more versatile adaptive immune system is required to clear the pathogenic organisms (Janeway et al. 2001, Kimbrell and Beutler 2001).

The adaptive immune system is distinctly different from the innate immune system in function, response, and structure. Evolutionarily the adaptive immune system is far more recent than the innate immune system (Kimbrell and Beutler 2001) and is thought to have evolved in jawed fish, roughly 500 million years ago, in part to recognize beneficial microbial species (Flajnik and Kasahara 2010). The adaptive immune system, is composed of several types of lymphocytes (white blood cells), which are able to traverse the body through an organism's blood and lymph circulatory systems (Hofmeyr 2001). Unlike the innate immune system, the adaptive immune system is able to 'learn' and adapt to target novel pathogens (Kimbrell and Beutler 2001). Should the adaptive immune system encounter the same pathogen repeatedly it can rapidly respond to and eliminate the pathogen, protecting an organism from re-infection (Janeway et al. 2001, Flajnik and Kasahara 2010). The adaptive immune system's ability to retain information pertaining to foreign pathogens is its main strength and defining feature.

When present, the innate and adaptive immune system collaborate in mounting a successful immune response (Kelly et al. 2007). The innate immune system serves as a fast-acting response to a foreign pathogen and can eliminate or contain the pathogen until the adaptive immune system activates a pathogen-specific response (Hofmeyr 2001). However, one of the biggest challenges the immune system faces is being able to distinguish between beneficial symbiotic microbial species in the gut and harmful pathogens (Lee and Mazmanian 2010).

Immune System and Microbiome Interactions

During early life stages the gut is sterile (Cash and Hooper 2005, O'Hara and Shanahan 2006, Neu 2007, Cho and Blaser 2012, Moloney et al. 2014), however through maternal and environmental routes microbes rapidly colonize the gut microbiome (Hooper 2004, Kelly et al. 2007, Smith et al. 2007, Round and Mazmanian 2009, Cerf-Bensussan and Gaboriau-Routhiau 2010, Spor et al. 2011, Cahenzli et al. 2013). During early life the gut microbiome and immune system collaborate in shaping both the microbiome community and immune system development (Cash and Hooper 2005, O'Hara and Shanahan 2006, Kelly et al. 2007, Round and Mazmanian 2009, Cerf-Bensussan and Gaboriau-Routhiau 2010, Mulder et al. 2011, Cahenzli et al. 2013, Thaïss et al. 2014, Zhang et al. 2014a). As an organism's gut becomes more developed and matures new niches proliferate, allowing for increased levels of microbial diversity and abundance (Spor et al. 2011). Initial colonizers along with additional microbial species which follow in sequential colonization events, establish communication with the host's immune system (Neu 2007). Cross talk between microbial species and the immune system allows the host's immune system to distinguish between pathogenic and mutualistic/commensal microbes (Kelly et al. 2007, Mulder et al. 2011). Once developed the gut microbiome and immune system cooperate to eliminate harmful pathogens within an organism (Kelly et al. 2007, Spor et al. 2011, Purchiaroni et al. 2013).

Evidence of environment-microbe-immune interactions was first proposed under the 'hygiene hypothesis', which states that early life exposure to microbes is essential for the proper development and maturity of an individual's immune system (Strachan 1989). Studies have demonstrated that infants born via Caesarian section often develop inadequate symbiotic relationships with colonizing microbes, leading to increased health issues including, a number of autoimmune disorders (e.g. allergies, asthma, irritable bowel disorder) later in life (Weng and Walker 2013, Dominguez-Bello et al. 2016).

The gut microbiome also provides an indirect health benefit for the immune system through colonization resistance. Colonization resistance refers to indigenous gut microbial species directly excluding pathogens from accessing resources via competitive exclusion (Dillion et al. 2005, Koch and Schmid-hempel 2011). The higher the microbial diversity in the gut, the harder it would be for a potentially harmful pathogen to acquire the necessary resources to establish itself within the community (Macpherson and Harris 2004, Dillion et al. 2005).

Perturbations to the establishment of the gut microbiome may result in further disturbances in immune system development, such as increased risk of inflammation, autoimmune diseases, and other immunological disorders (Round and Mazmanian 2009).

Experiments Using Germ-Free Mice

The effects of the absence of a gut microbiome can be determined using germ-free mice, making them valuable in determining specific bacteria-host interactions. Germ-free mice have increased susceptibility to bacterial, virus, and parasitic infections (Sprinz et al. 1961, Maier and Hentges 1972, Round and Mazmanian 2009). Germ-free mice have also been shown to have underdeveloped immune system traits such as mucus thickness (Sharma et al. 1995), invariant natural killer cells (Olszak et al. 2012), Peyer patches, lymphoid follicles (Round and Mazmanian 2009), and IgA secretion (Smith et al. 2007, Chung and Kasper 2010). In previous studies, germ-free mice infected with *Shigella flexneri* demonstrated decreased immune responses as well as increased mortality compared to conventionally colonized mice (Sprinz et al. 1961, Round and Mazmanian 2009).

The immune system of germ-free mice can be partially restored when colonized by microbial species (Xu and Gordon 2003, Mulder et al. 2011). When colonized by *B. thetaiotaomicron* previously germ-free mice initiated angiogenesis - a process carried out by the

microbial-sensing Paneth cells (innate immune system) - resulting in an increased expression of *Ang4*, a bactericide that targets pathogens (Stappenbeck et al. 2002). Research on microbiome-immune interactions carried out on germ-free mice suggest that immune system development is only partially encoded in an organism's DNA and that for an immune system to fully develop it must rely on exposure and interactions with beneficial symbionts from extrinsic environments (Xu and Gordon 2003, Hooper 2004, Cash and Hooper 2005, Kelly et al. 2007, Smith et al. 2007, Round and Mazmanian 2009). Functional evidence of this has been provided by Abolins et al. (2011), who reported that conventionally colonized wild mice exhibited a stronger immune system response and baseline levels, than germ-free mice.

Environment and Microbiome Interactions

Early and continuous exposure to highly diverse environmental microbiota are expected to be necessary for the establishment of a fully developed and stable gut microbiome (Hooper 2004, Mulder et al. 2011). Previous research has shown different microbiomes within an organism can be affected by exposure to different environmental factors, such as maternal influences (Lucas and Heeb 2005, Friswell et al. 2010), diet (Ley et al. 2008a, 2008b, Wang et al. 2014, Maurice et al. 2015), and biogeography (Lankau et al. 2012, Linnenbrink et al. 2013). Environmental influences may result in distinct enterotypes between populations of the same species and can be sources of inter- and intra-individual variance in gut microbiome composition (Spor et al. 2011).

Maternal Influences on the Gut Microbiome

Mammals are first exposed to environmental microbes as they pass through the birth canal (Hooper 2004, Kelly et al. 2007, Smith et al. 2007, Round and Mazmanian 2009, Cerf-Bensussan and Gaboriau-Routhiau 2010, Mulder et al. 2011, Spor et al. 2011, Cahenzli et al. 2013). Juveniles may also engage in coprophagy during early life stages, whereby microbes may be

vertically transmitted, furthering offspring exposure to beneficial gut microbes (Lucas and Heeb 2005, Ley et al. 2008b, Friswell et al. 2010, Spor et al. 2011, Kohl et al. 2017).

Friswell et al. (2010) were able to demonstrate the effects of maternal influences among mice, by implanting female mice with embryos of different inbred lab strains. After initial colonization events occurred the gut microbiome of juvenile mice resembled that of the surrogate mother (Friswell et al. 2010), providing evidence that maternal influences affect gut microbiome composition.

Although Friswell et al. (2010) provided evidence for maternal influences on the gut microbiome, Lucas and Heeb (2005) demonstrated that in the wild, maternal effects may be masked by more prominent environmental influences. Lucas and Heeb (2005) assessed the cloacal microbiome composition of nestling blue tits (*Parus caeruleus*) and great tits (*Parus major*), which had been fostered by a surrogate mother from the opposite species. Nestlings were extracted from their nest of origin at 3-4 days of age (already possessing a microbiome) and fostered for fourteen days before being returned to their original nest. After the fourteen days, the microbiome of cross-fostered individuals were more similar between individuals in the same nest than biological siblings, suggesting that although maternal effects may be present, they may be quickly masked by alternate environmental exposures shortly after birth (Lucas and Heeb 2005).

Diet and the Gut Microbiome

Changes in diet can be an important driver for the evolution of new species and although yet unexplored thoroughly, it is thought that gut microbiome communities had the ability to facilitate the evolution of new species (Ley et al. 2008a). A study examining sixty different mammalian species showed that microbiome composition was heavily affected by diet (Ley et al. 2008a). Through network-based analysis, Ley et al. (2008a) showed that gut microbial

compositions of hosts from the same species were more closely related to each other than that of different species. When examining different species, their resemblance to one another was shown to be correlated with diet (Ley et al. 2008a, e.g. herbivores, omnivores and carnivores).

In a laboratory setting Wang *et al.* (2014) demonstrated that when mice from two geographically different wild populations were housed in a laboratory setting for a year their enterotypes converged, demonstrating the strong affect dietary factors can have on microbial composition. Maurice et al. (2015) was also able to detect an association between the gut microbiome of wild mice and dietary changes when examining the gut microbiome of wild wood mice (*Apodemus sylvaticus*) changed over the course of two years. Changes in the gut microbiome of mice was shown to mirror dietary shifts caused by seasonal influences (Maurice et al. 2015). While dietary changes may affect local-scale changes in the gut microbiome, large-scale changes have shown to relate to biogeography (Lankau et al. 2012, Linnenbrink et al. 2013).

Biogeography and the Gut Microbiome

Gut microbiome communities represent a collection of microbes that individuals are exposed to and therefore have the potential to be shaped by meta-community dynamics on both local and regional scales (Lankau et al. 2012). Biogeographic influences on gut microbiome composition have been shown to be present in land and marine iguanas (*Conolophus pallidus* and *Conolophus subcristatus*, respectively, Lankau et al. 2012) in the Galapagos, as well as house mice (*Mus musculus*, Linnenbrink et al. 2013) in Europe. Lankau et al. (2012) and Linnenbrink et al. (2013) provided evidence that geographic location influences the similarity of the gut microbiome composition between populations.

Lankau et al. (2012) provided evidence for geographic influences within a meta-community as well as within a single population, suggesting that the gut microbiome composition

of individuals is influenced not only by the local (e.g. water, food and soil) microbial community, but by the regional (i.e. between island populations) microbial community as well. Assemblage of gut microbial species among Galapagos Islands may be explained via meta-community dynamics such as patch-dynamic models or neutral ecological drift (Lankau et al. 2012). Linnenbrink et al. (2013) found that geographic distance was the main variable in determining the gut microbiome of house mice in Europe. Variation in microbiome communities was noticeable when looking between sample sites (regional scale), however, variation was not noticeable when looking within sample sites (local scale, Linnenbrink et al. 2013). Similar to Lankau et al. (2012), Linnenbrink et al. (2013) suggested that the variation in regional microbiome differences is likely due to neutral dispersal limitations.

Effect of Captivity on Gut Microbiome Composition

Organisms in captivity have repeatedly been shown to possess different and typically less diverse gut microbiome communities than their wild counterparts (Uenishi et al. 2007, Villers et al. 2008, Xenoulis et al. 2010, Nelson et al. 2013, Cheng et al. 2015). These differences may be due to captive animals being confined to artificial environments, resulting in different diets, behaviour, and social interactions (Nelson et al. 2013). Previously discussed environmental factors, maternal influences, diet, and biogeography may all likely contribute to the differences seen in the gut microbiome of captive versus wild individuals. Animals in captivity exhibit lower gut microbiome diversity levels than their wild counterparts, which may lead to health and fitness consequences resulting in animals being ill-equipped for reintroductions (Redford et al. 2012). Gut microbiome communities contain important symbionts for a number of species and should be considered in future conservation programs targeting captive animals for reintroductions (Redford et al. 2012).

Current Environmental and Microbiome Perspectives

Environmental influences, such as maternal effects (Lucas and Heeb 2005, Friswell et al. 2010), diet (Ley et al. 2008a, 2008b, Wang et al. 2014), biogeography (Lankau et al. 2012, Linnenbrink et al. 2013) and captivity (Nelson et al. 2013, Cheng et al. 2015) all have an impact on gut microbiome composition. Studies comparing the overlap between gut microbiome composition of organisms with microbial species found directly in the environment (e.g. on food sources, in seawater), however have failed to find a significant similarities (Costello et al. 2014, Kohl and Dearing 2014). One possible explanation is that gut microbiome communities are influenced by a multitude of environmental influences and that it is unlikely that a single environmental variable (e.g. single prey species, water, or soil sample) alone will have a large impact an organism's gut microbiome.

Gut Microbiome Composition: Of Mice and Mammals

Mammals have relatively homologous gut microbiome diversity at high taxonomic levels (i.e Phylum), however, microbial diversification between mammalian species increases at lower taxonomic levels (Cho and Blaser 2012). Gut microbiome composition in mammals is dominated by Bacteroidetes and Firmicutes, with smaller influences from Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria at the phyla level (The Human Microbiome Project Consortium 2012, Sommer and Bäckhed 2013). Despite being similar at the phylum level, at the genus level 85% of the microbial species found in mice are not detected in humans (Ley et al. 2005). While large scale results from previous research may be more easily extrapolated between species, caution should be taken when attempting to extrapolate fine scale results between different species or even distinct populations. As taxonomic distance increases, further caution should also be applied towards attempting to extrapolate results between species.

Study Species

The majority of studies currently in the literature use in-bred lab mice as their study species, whereas microbiome studies relating to wild populations are underrepresented in the current literature (Hird 2017). The few studies that have used wild mice, have all been conducted in Europe using house mice (Linnenbrink et al. 2012) or wild wood mice (*Apodemus sylvaticus*, Maurice et al. 2015). Using wild deer mice will allow this study to compare findings to a related species and the existing knowledge pertaining to the gut microbiome composition of wild populations.

The deer mouse (*Peromyscus maniculatus*) is a small terrestrial, nocturnal rodent found throughout forests in North America (Banfield 1974). Deer mice are the most abundant small mammal in North America and are easily captured using live traps. More importantly deer mice are easy to temporarily house in a laboratory setting (Banfield 1974), allowing for a larger sample size and more robust statistical analysis to be performed. Deer mice territories overlap between individuals and are usually limited to 10,000 m² for males and slightly less for females, increasing the ability to successfully recapture individuals (Banfield 1974).

Deer mice have multiple oestrous cycles between March and October, with gestation occurring in just twenty-two to thirty-five days (Banfield 1974). The average number of pups per litter in deer mice is 4.04 (Banfield 1974). Female-juvenile mice become sexually mature around thirty-two to thirty-five days after birth, allowing a juvenile mice to have their own litter the same season they are born.

Chapter one: Environmental influences on α - and β -diversity of gut microbiome communities in deer mice (*Peromyscus maniculatus*)

Factors contributing to the assemblage of gut microbial communities are poorly understood. The purpose of this chapter was to determine how the extrinsic environment

contributes to the development of gut microbiome communities (GMCs). To test this I analyzed the gut microbiome communities of deer mice born in captivity versus the wild, as well as the changes that occurred in gut microbiome communities due to translocations, between wild and captive environments. I hypothesized that gut microbiome communities are strongly influenced by exposure to microbes located in the exogenous environment. It is predicted that GMCs will display unique characteristics based on an individual's surrounding environment. Deer mice born in the same environment (captivity or wild habitat) should have more similar GMC α -diversity and compositions, compared to deer mice born in a different environment. GMCs (α - and β -diversity) of deer mice translocated to different environments are expected to homogenize with deer mice that inhabit the same environment. Additionally, maternal effects were hypothesized to occur during the initial capture phase, with GMCs of littermates being distinctively more similar to each other compare to deer mice from alternate litters. Maternal influences however are expected to disappear post-translocation due to extrinsic environmental influences masking maternal influences. Deer mice born in captivity are expected to have more similar GMCs to their siblings rather than counterparts from other litters or wild-caught individuals, however post-translocation unique enterotypes are not expected to exist within litters. Lastly, this chapter will also examine sex differences in gut microbiome communities between individuals born in the wild and a captive environment.

Chapter two: Relationships between gut microbiome α -diversity and host immunocompetence

Chapter two examined relationships between α -diversity within gut microbiome communities and the magnitude of an individual's immune response. I hypothesized that gut microbiome α -diversity influences an individual's immunocompetence. Individuals with higher levels of α -diversity are expected to possess optimal haematological conditions (i.e. optimal [i.e.

population mean] hematocrit ratios, increased white blood cell and neutrophil:lymphocyte ratios) as well as higher bacteria killing ability (BKA) as a result of increased microbial exposure, during early life stages. Results from this chapter are valuable to captive breeding programs, whose animals have often been seen to have lower levels of gut microbiome α -diversity than their wild counterparts. Specific microbes which were found to be important in differing enterotypes in *Chapter 1* were also examined to determine their relationship with an individual's immunocompetence.

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Chapter 1: Environmental influences on α - and β -diversity of gut microbiome communities in
deer mice (*Peromyscus maniculatus*)

1.1 Abstract

Vertebrate gastrointestinal tracts host trillions of commensal, pathogenic, and beneficial symbiotic microbes which have co-existed with their hosts over millions of years. These microbial communities, mainly composed of bacteria, provide their host with numerous health benefits and may play a substantial role in the conservation success of their hosts. However, factors contributing to the assemblage of these microbial communities are poorly understood. The purpose of this study was to determine how the extrinsic environment contributes to the development of gut microbiome communities (GMCs). Deer mice (*Peromyscus maniculatus*) raised in captivity and in the wild, had fecal samples collected at approximately three weeks of age. Additional samples were collected two weeks later, with some individuals being translocated between captive and the wild environments. Microbial data was analysed using 16S rRNA gene next-generation Illumina HISEq sequencing methods. GMCs of deer mice were more closely related between neighbours who shared the same environment, regardless of where an individual was born (i.e. captivity or the wild), demonstrating that GMCs are heavily influenced by the extrinsic environment and can rapidly change over time. Mice in natural environments contained more diverse GMCs with a higher abundance of Firmicutes compared to captive individuals, with evidence suggesting that *Ruminococcaceae*, *Helicobacteraceae* and *Lachnospiraceae* spp. abundances are dependent on exposure to the natural environment. Future studies should examine the health implications of extrinsically dependent microbes identified in this study to further understand environment-microbe-host evolutionary and ecological relationships.

1.2 Introduction

Microbial communities inhabiting the gastrointestinal tract of vertebrates have co-existed over millions of years, forming pathogenic, commensal, or even mutualistic relationships (Xu and Gordon 2003, Hooper 2004, Bäckhed et al. 2005, O'Hara and Shanahan 2006, Manson et al. 2008, Round and Mazmanian 2009, Hooper et al. 2015). These microbial communities may affect their hosts by influencing metabolic functions, brain development, nutrition extraction, and immune system development (Stappenbeck et al. 2002, Cash and Hooper 2005, Dillion et al. 2005, Round and Mazmanian 2009, Moloney et al. 2014), however factors influencing the assemblage of microbial communities are poorly understood.

Gut microbiome communities (GMCs) of captive populations have been shown to contain less diverse communities compared to wild populations (Villers et al. 2008, Xenoulis et al. 2010, Nakamura et al. 2011, Kohl et al. 2014, Cheng et al. 2015), however, this trend does not appear to be universal (Nelson et al. 2013, McKenzie et al. 2017). Differences in GMC composition between captive and wild individuals may be due to a number of extrinsic environmental factors, including, diet (Ley et al. 2008a, Muegge et al. 2011, Wang et al. 2014), biogeography (Lankau et al. 2012, Linnenbrink et al. 2013), host richness (Gavish et al. 2014, Adair and Douglas 2017), and time (Maurice et al. 2015, Bobbie et al. 2017). Microbes acquired through consumption of food contribute to the pool of microorganisms an individual draws upon to form its GMC. Dietary analyses of mammalian species has demonstrated that organisms possessing similar diets (e.g. herbivore, omnivore, carnivore) possess distinctly similar GMCs, referred to as enterotypes (Ley et al. 2008a, Groussin et al. 2017). As well, alterations (Wang et al. 2014) and seasonal shifts (Maurice et al. 2015) in diet have been shown to cause sequential changes in GMCs. Individuals may also acquire microorganisms from environmental sources of microbial congregation (i.e. soil,

water, contact with other hosts [Lee et al. 2010, Gavish et al. 2014]), which are subjected to change over different biogeographic regions. On local and regional levels GMCs behaved similarly to meta-communities constrained by neutral dispersion limitations (Langenheder and Székely 2011, Lankau et al. 2012, Linnenbrink et al. 2013), indicating that microbes have equal opportunities at successfully colonizing an environment; the more exposure a host has to a microbial species the more likely that species is to persist in the host's GMC (Hubbell 2001, Chave 2004). Microbial species pools that hosts draw upon, however, are not static and therefore change over time, inducing temporal changes in GMCs (Maurice et al. 2015, Adair and Douglas 2017, Bobbie et al. 2017). Numerous extrinsic and intrinsic temporally dynamic factors including changes in: food source and availability, parasite load, and intrinsic host physiology (i.e. torpor, reproductive condition) have also been shown to influence GMC composition, indicating that a single sample may simply be catching a 'snap-shot' of a particular GMC (Baxter et al. 2015, Maurice et al. 2015, Bobbie et al. 2017).

Maternally transferred microorganisms pioneer the colonization of an individual's GMC upon passing through the birth canal (Benson et al. 2010, Friswell et al. 2010, Spor et al. 2011, Funkhouser and Bordenstein 2013). For example, lab-strain mice prenatally transplanted into surrogate mothers were shown to share homologous GMCs with their surrogate siblings, rather than genetically related siblings (Friswell et al. 2010). Friswell et al. (2010) demonstrated that the initial colonization phase that occurs when passing through the birth canal can have a strong initial influence on the gut microbiome during early life stages. Maternal influences during early life stages were also demonstrated in wild populations of post-natal fostered nestling birds (*Parus caeruleus* and *P. major*), where the cloacal microbiome of fostered nestlings was shown to be more similar to individuals in shared nests rather than host species (Lucas and Heeb 2005).

The influence of sex on GMCs is not completely understood, but wild populations of model species have demonstrated important sex-diet-gut microbiome interactions that affect the composition of GMCs (Bolnick et al. 2014). Alternatively, lab-based studies have shown in mice (inbred lab strains), that sex differences are insignificant or only explain a small amount of variation between mice GMCs (Kovacs et al. 2011, Campbell et al. 2012).

The main disadvantage of sterile and laboratory based studies is that the application to wild populations is limited (Kohl and Dearing 2014, Kreisinger et al. 2014). Therefore such studies should be paired with analysis of populations under natural conditions before results are extrapolated to wild populations. Relatively little research has been applied towards understanding the formation of GMCs with wild populations (Hird 2017), which is surprising because GMC composition has been linked with host-health. Understanding the establishment and temporal changes of GMCs, may reveal important host-microbe interactions and information regarding the conservation of vertebrate hosts (Redford et al. 2012).

This study investigates how environmental factors influence the composition of GMCs. Deer mice (*Peromyscus maniculatus*) were used in this study to examine how GMCs differentiate between individuals born and reared in captivity (first generation) versus the wild, as well as the consequences of translocating individuals from captivity to the wild and vice versa. The short gestation period (22-35 days), abundance, and ability to easily translocate deer mice makes them an ideal study species (Banfield 1974). In-depth classification of environment-linked microbes will also allow for an increased understanding of evolutionary and ecological interactions between the environment, GMCs and their host, a topic currently underrepresented in the literature (Hird 2017).

It is hypothesized that gut microbiome communities are strongly influenced by exposure to microbes located in the exogenous environment. Subsequent predictions state that GMCs would display enterotypes based on an individual's surrounding environment. Deer mice born in the same environment (captivity or wild habitat) should have more similar GMC α -diversity and compositions, compared to deer mice born in a different environment. GMCs (α -diversity and composition) of deer mice translocated to different environments were expected to homogenize with deer mice that inhabit the same environment. GMCs were also analysed to determine which microbes (i.e. specific operational taxonomic units [OTUs]) were strongly associated with captive and wild environments. Maternal effects were hypothesized to occur during the initial capture phase, with the GMCs of littermates being distinctively more similar to each other compared to offspring from other females. Maternal influences however were expected to disappear post-translocation due to extrinsic environmental influences masking maternal influences. Deer mice born in captivity were expected to have more similar GMCs to their siblings rather than counterparts from other litters or wild-caught individuals, however post-translocation unique enterotypes were not expected to exist within litters.

1.3 Methods

All methods in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Laurentian University, protocol number 2016-03-01.

1.3.1 Study site and sample collection

Deer mice (*Peromyscus maniculatus*) were captured between May – August 2016 in Algonquin Provincial Park, Ontario, Canada (45°35'N, 78°31'W) using Longworth live traps (Rogers Manufacturing Co., Canada). Animals were trapped on a 220m x 430m grid in an area dominated by sugar maple (*Acer saccharum*). Traps were set just prior to sunset (18:00-20:00h

depending on the time of year) and were checked during the morning, starting half-an-hour before sunrise (04:45-06:00 depending on the time of year). Each trap was provisioned with dry polyester bedding for insulation as well as baited with sunflower seeds that had been soaked in water the day before. Captured individuals were provided with unique alphanumeric ear tags (National Brand and Tag co., Newport, KY) for future identification, sexed, and assessed for reproductive condition. Deer mice in the wild were determined to be juveniles based on pelage (i.e. grey) and weight (<14 g), at which point deer mice are expected to be between three to five weeks of age (Banfield 1974).

Fecal samples have previously been demonstrated as being appropriate for examining GMCs and are comparable to aseptically collected samples (Kohl et al. 2015). To understand how extrinsic environmental factors influenced GMCs, faecal samples were collected from captive and wild born individuals. Fecal samples were taken directly from the anus of deer mice upon capture, limiting risk of contamination. When necessary, fecal collection was aided by the use of sterile tweezers. Fecal samples were stored in Eppendorf tubes, put on ice in the field and then placed in -20°C freezer until analyzed.

1.3.2 Experimental design

Pregnant dams were captured in May and June. Once a female was determined to be pregnant (judged by assessing weight, body shape and palpations), it was transferred to, and housed in, a field-laboratory setting. The offspring of these dams would serve as the captive-raised individuals of the experiment.

This study consisted for four main experimental groups; Wild-Wild (W-W), Wild-Captive (W-C), Captive-Wild (C-W), Captive-Captive (C-C) (**Figure 1-1**). Group names correspond to where an animal was born (first part of name) as well as where an individual was placed during

the post-translocation phase of the experiment. Each deer mouse was sampled twice. Deer mice born in captivity were first sampled at three weeks of age, when deer mice would have been weaned from their mothers. After the initial sample had been acquired all but two deer mice from each litter ($\bar{x} = 4.5$ juveniles/litter) in captivity were translocated (i.e. C-W group) to the wild (same grid where wild born individuals were captured), while the remaining deer mice stayed in captivity (i.e. C-C group). Two weeks after the initial sample was acquired an additional sample was taken from all captive born deer mice (provided that individuals translocated to the wild were recaptured). Juvenile deer mice born in the wild were sampled upon capture. A random subset of deer mice born and captured in the wild were translocated to captivity after initial samples had been taken (i.e. W-C group), while the remaining individuals remained in the wild (i.e. W-W group). Similar to the captive born individuals, two weeks after wild born juvenile deer mice were initially sampled, they were sampled again.

Deer mice translocated from captivity to the wild were transferred in wooden nest boxes (12.5 cm x 12.5 cm x 15 cm) filled with bedding material and sunflower seeds. Nest boxes were attached to a tree at chest height at the location where the dam was originally captured. Exit holes were exposed after the nest box was firmly attached to the tree so that individuals could exit freely. Non-translocated captive and wild individuals served as controls (C-C, and W-W groups, respectively). Deer mice who remained in captivity (C-C group) were co-housed (two individuals per cage), while deer mice translocated into captivity from the wild (W-C group) were provided with their own cage to avoid potential aggressions between unrelated individuals.

1.3.3 Captivity conditions

Dams were housed in separate cages (Plexiglas, 35cm x 15cm x 17cm) in the same room. Each cage was provided with corn-cob bedding (Pestell Pet Products Easy Clean Corn Cob

Bedding), nesting materials (Anderson's CN Crink-I'nest™ and Ancare Nestlets) as well as environmental enrichment. The day-night cycle mimicked natural conditions with lights being turned on half-an-hour after sunrise and half-an-hour before sunset (exact times varied depending on natural sunrise/sunset times). The temperature of the lab reflected the outside temperature, unless the temperature dropped below 5°C, at which point the room was heated to 15°C. Individuals were provided with water and food (8640 Tekland Laboratories) *ad libitum*. All cages had equal access to natural light entering the field laboratory and all cages/individuals were treated identically. Food, water bottles, and cages were handled with sterile gloves to avoid contamination with human associated microbes. Cages, water bottles, and environmental enrichment material were cleaned using diluted bleach on a weekly basis. It is important to note that laboratory conditions were meant to mimic those of captivity rather than a sterile germ-free setting.

1.3.4 Microbial Extractions

Microbial extractions were performed from fecal samples collected during initial and post-translocation sampling phases. Therefore each deer mouse had two fecal samples processed. Extractions were conducted using QIAamp DNA Stool Mini Kit (Qiagen, Mississauga, ON, Canada; #51504), following the manufacturer's Isolation of DNA from Stool for Pathogen Detection instructions. After the extraction process DNA purity was quantified via spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA), to assess for quality of DNA by examining the 260/280 wavelength ratio. Samples whose 260/280 ratio were between 1.8 – 2.0 had high DNA quality, while samples outside that range were designated as low DNA quality samples. PCR was performed on low quality sample to ensure that they would amplify prior to sequencing. Samples were stored at -20°C until sequenced for approximately 3-weeks.

1.3.5 PCR

Samples of poor DNA purity underwent PCR to determine if these samples could be amplified prior to sequencing. The bacterial 16s rRNA gene were PCR amplified for poor quality samples using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTAC-CTTGTTACGACTT-3') primers. Each 20 µl reaction contained 1 µl of template DNA, 0.5 µl of forward and reverse primers (10 µM), 0.5 µl of dNTP (25 mM each), 1.5 µl MgCl₂ (25 mM), 2.5 µl 10x PCR buffer, 13.4 µl distilled H₂O and 0.125 µl (5 U/µl) AmpliTaq® DNA polymerase. Thermocycling conditions were 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min, final extension was 72°C for 10 min. PCR products were then evaluated using agarose gel electrophoresis (1.0 g agarose, 70 ml TAE buffer and 3 µl gel stain). Samples that still failed to amplify were concentrated using SpeedVac-Centrovap (~2hr) and then re-run through the same PCR process described above.

1.3.6 Amplicon sequencing

Samples were sequenced using Illumina MiSeq system (Illumina Biotechnology Co., San Diego, USA) by Metagenom Bio Inc. (Toronto, Canada), using their designated protocol. Broad prokaryote primers were used to target the V3-V4 regions of the bacterial 16S RNA gene, Pro341F (5'-C-CTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATC-C-3'). Before being pooled samples had an index sequence of 6 bp incorporated, allowing for sequencing to be done in a single run. A 25 µl PCR reaction was conducted using the following conditions: 5 µl of standard OneTaq buffer (5x), 0.25 µl of 25 mM dNTP, 0.5 µl of both primers, 1 µl BSA (12mg/ml), 0.125 µl of OneTaq DNA polymerase (New England Bio, MA, USA), 1-10 ng DNA and water to reach the necessary 25 µl volume. PCR thermocycling conditions were set at: 94°C for 5 mins (initial denaturation), followed by 30 cycles of 94°C for 30 sec (denaturation), 53°C for 45 sec (annealing), 68°C for 1 min (extension), and finished with a final extension at 68°C for 10

minutes. PCRs were performed in triplicate for all samples with finished products being checked on 2% agarose gel. Bands were then excised using a MinElute gel extraction kit (Qiagen, Hilden, Germany). Purified DNA libraries were quantified via Qubit with the dsDNA HS assay kit (Life Technologies, CA, USA), with library pools spiked with 5% phix control (V3, Illumina) to improve base imbalance. Paired-end sequencing with 250bp read lengths were conducted using MiSeq Reagent kit V2 (2 x 250 cycles) on an Illumina MiSeq platform.

1.3.7 Bioinformatics

Sequencing data were processed using a series of open-source bioinformatics tools. PEAR (Zhang et al. 2014; v0.9.8) was used to pair and filter sequences to 390-590 base pairs. Primers were then trimmed from each sequence (Brian 2014; v37.09). USEARCH8 (Edgar 2010; v10.0.240) was used to de-replicate and sort (by abundance) sequences as well as removing any singletons and doubletons. Singletons and doubletons were removed from the dataset prior to statistical analysis to provide more accurate results. Through QIIME (Caporaso et al. 2010; v1.9.1) Operational Taxonomic Units (OTUs) were clustered using UPARSE (97% similarity), which included a chimera check step and then mapped specific OTUs. Sequences were referenced against the GreenGenes (97% confidence cut-off) database to assign taxonomic classification.

1.3.8 Statistical Analysis

All statistical analyses were conducted in R (R Core Team 2014) [v3.3.1]) using RStudio (R Studio Team 2015 [v1.0.136]), vegan (Oksanen et al. 2015 [v2.4-3]), phyloseq (McMurdie and Holmes 2013b [v1.16.2]), ggplot2 (Wickham 2009 [v2.2.1]), and Deseq2 (Love et al. 2014 [v1.12.4]) packages. Variance stabilizing transformations (VSTs) were used to normalize GMC data. VSTs reduce the risk of false positives and do not discard available data, while providing

increased accuracy in determining OTU abundances, compared to the traditionally used method of rarefaction (McMurdie and Holmes 2013).

Methods outlined here were carried out for both initial and post-translocation capture periods. Chao1, Fisher, and Shannon's indices were used to assess α -diversity scores. Different α -diversity measurements incorporate different algorithms and therefore can be more or less sensitive to rare OTUs. Using multiple α -diversity measurements provides, an increased understanding of how confidently results can be interpreted as well as more opportunity for results to be compared between studies. There are a number of difficulties involved with the comparison of microbiome studies including the use of different samples sites, primers, and normalizations techniques (e.g. rarefaction, DeSeq2), therefore the use of multiple α -diversity indices may limit further, unnecessary limitations for between study comparisons. However, it is important to recognize the strengths and weaknesses of different diversity indices. Although commonly used Shannon's diversity index can be problematic when dealing with bacterial communities, due to the emphasis placed on rare species (Hill et al. 2003). Additionally, Shannon's diversity index can overestimate diversity levels when sample sizes are small. Comparatively, the Chao1 α -diversity index (non-parametric) calculates richness based on asymptote of a species accumulation curve (Kohl et al. 2015), has been shown to work particularly well with microbial datasets (Hughes et al. 2016). Fisher's α -diversity index, which incorporates log-series fitted species abundance curves (Bonilla-Rosso et al. 2012), has also previously been used successfully to assess microbial communities (Gavish et al. 2014). Although Shannon's index may not be the most effective in accurately examining large microbial communities, it does possess the advantage of incorporating evenness, while Chao1 and Fisher's index focus mainly on richness. For Chao1 and Fisher α -diversity indices ANOVA and post-hoc Tukey tests were used to determine differences in α -

diversity levels between all four experimental groups, during initial and post-translocation sampling phases. Due to the non-normality of data for Shannon's diversity indices, Kruskal-Wallis and post-hoc Dunn's test were used to test for significance.

A mixed-effect ANOVA (with Tukey post-hoc) model was used to assess differences in α -diversity (when using Chao1, and Fisher's indices) within each group, between the initial and post-translocation sampling periods, to determine if there were significant changes over the two-week experimental phase. Similar to previous methods, Kruskal-Wallis and Dunn's post-hoc test were used when using Shannon's diversity index.

Turnover refers to the difference in community abundance (i.e. number of individuals within a community) between two different communities or time periods. Turnover was calculated for each deer mouse GMC based on comparing differences (absolute values) in OTU abundances between initial capture and post-translocation capture samples. An ANOVA and Tukey post-hoc analysis was used to determine significant differences between GMC turnover rates based on the four different experimental groups.

β -diversity measurements including Bray-Curtis, Unweighted and Weighted UniFrac metrics (Caporaso et al. 2010), were used to assess differences in gut microbiome communities between individuals as well as maternal and sex effects. The Bray-Curtis dissimilarity measurement examined the abundance of different species within communities, while ignoring phylogenetic relatedness. UniFrac measurements assess the amount of divergence between OTUs to determine if there are differences in GMCs based on the lineages they contain, with weighted measurements also incorporating abundances (Lozupone and Knight 2005). Statistical differences between group GMCs and litters (for captive born deer mice), were tested together using ADONIS (permutational MANOVA) models, with 9999 permutations and reported F , R^2 and p values, to

determine if there were significant differences between captive and wild GMCs during the initial and post-translocation phases. Principal coordinate analysis (PCoA) ordinations were used to visually display differences within GMCs, under different β -diversity measurements. The same approach was used to test for sex differences, however, a separate analysis was conducted for captive and wild born individuals.

GMC differences between sampling periods and experimental groups were determined by evaluating changes in microbial families; microbes which made up 1% or more of an individual's GMC. Family was the lowest classification level that provided accurate results. Further classification (i.e. genus and species level) resulted in the vast majority of OTUs being unidentifiable. Mann-Whitney U tests were used to compare differences in GMCs for captive-born and wild-born deer mice during the initial capture period. Kruskal-Wallis and post-hoc Dunn tests were used to determine significant differences in microbial families between the four experimental groups post-translocation. Paired Wilcoxon tests were then used to assess changes in microbial families within each experimental group.

To account for effect size, a linear discriminant analysis (LDA) of effect size (LEfSe) analysis (Segata et al. 2011) was used to determine which taxonomic groups were associated with the GMCs of captive/wild (initial capture), and C-W as well as W-C deer mice GMCs post-translocation. LEfSe analysis allows for differences between GMCs to be determined using Kruskal-Wallis ($\alpha=0.05$) tests while also incorporating biological consistency and effect size (Segata et al. 2011).

Constrained Analysis of Principal Coordinates (CAP) analysis was used to determine the top ten most enterotype-defining OTUs (i.e. OTUs that are characteristic of a unique type of microbiome [Gorvvitobskaya et al. 2016]) for captive and wild enterotypes (initial capture) as well

as each group post-translocation. Bray-Curtis dissimilarity measurements were used in the CAP analysis, allowing abundance to be incorporated into determining which OTUs were enterotype-defining. Therefore, OTUs that were determined to be enterotype-defining were both highly abundant as well as influential in GMCs of deer mice. Changes in the relative abundance of enterotype-defining OTUs in both initial and post-translocation capture periods for captive and wild GMCs, were analysed through paired Wilcoxon signed-rank tests for individuals in each group.

1.4 Results

3,235 unique OTUs were present among all deer mice, with individuals on average containing 444 ± 114 (standard deviation) unique OTUs. In total, 69 individuals were included in the initial capture phase and were designated as belonging to either the captive or wild groups ($n=36$ captive; $n=33$ wild) based on where they were born. Fecal samples from 34 deer mice were obtained during the post-translocation sampling period of the experiment: 8 captive-captive (C-C), 5 captive-wild (C-W), 13(8) wild-captive (W-C) and 8 wild-wild (W-W) were collected. All deer mice were sampled twice (i.e. initial and post-translocation phase), except in the W-C group where only 8 of the 13 were sampled twice. The additional five deer mice in the W-C were used for post-translocation comparisons between groups, but not for looking at changes within the group since they did not have fecal samples from the initial sampling phase.

GMCs of captive born individuals showed marginal differences between males and females (Weighted UniFrac: $F = 1.81$, $R^2 = 0.051$, $p = 0.10$). Sex differences between GMCs of wild deer mice showed similar results, with only marginal differences being present (Weighted: $F = 1.77$, $R^2 = 0.054$, $p = 0.10$). However, sex only explained 5.1% and 5.4% of the differences between male and female GMCs in captive and wild born deer mice, respectively, suggesting that sex differences

have a limited influence on GMC variation, therefore sexes were pooled in subsequent statistical analysis.

1.4.1 α -diversity

Individuals born in the wild (W-C and W-W groups) had significantly higher levels of α -diversity than individuals born in captivity (C-C and C-W groups) during the initial sampling period (Chao1: $F_{1,3} = 32.23$, $p < 0.01$; Fisher: $F_{1,3} = 28.07$, $p < 0.01$ [**Figure 1-2**]). Shannon's index showed similar results, however, did not detect a difference between the C-W and W-C groups ($\chi_{1,3} = 19.08$, $p < 0.01$).

Significant differences were present between α -diversity levels of the four experimental groups post-translocation (Chao1: $F_{1,3} = 4.11$, $p < 0.05$; Fisher: $F_{1,3} = 5.07$, $p < 0.01$, Shannon: $\chi_{1,3} = 10.44$, $p < 0.05$ [**Figure 1-3**]). Post-translocation W-W individuals had higher α -diversity scores compared to W-C counterparts (Chao1: $p = 0.069$; Fisher: $p < 0.01$; Shannon's: $p < 0.05$). Significant or marginal differences were maintained between the control groups C-C and W-W (Chao1: $p = 0.01$; Fisher: $p < 0.05$; Shannon's: $p = 0.055$). No significant difference in α -diversity was seen between C-C and C-W groups, or between the translocated (C-W and W-C) groups. C-W deer mice showed an increase in α -diversity after being translocated to the wild, however their diversity scores were only similar to W-W deer mice when using Shannon's diversity index, under Chao1 and Fisher's index C-W deer mice failed to achieve similar α -diversity levels as W-W post-translocation.

During the post-translocation sampling period W-W deer mice contained 519 (21.3% of all OTUs among deer mice sampled post-translocation) unique OTUs, over three times more than either C-C (124 unique OTUs), C-W (167 unique OTUs), or W-C (145 unique OTUs) (**Figure 1-4**).

When examining differences that occurred within groups between sampling periods (**Figure 1-5**) a significant increase in α -diversity was seen for C-W group under Chao1 ($t_{1,24} = 4.23, p < 0.01$), however no significant differences were seen when using Fisher's or Shannon's diversity indices. W-C group showed a significant decrease under Chao1 ($t_{1,24} = -4.61, p < 0.01$), and Fishers ($t_{1,24} = -4.09, p < 0.01$), with only a marginal decrease when using Shannon's diversity (Kruskal-Wallis rank sum test, $p = 0.080$). Control groups C-C and W-W did not show any significant changes in α -diversity between sampling periods, suggesting that diversity did not significantly change over two weeks when their environment remain the same.

Significant differences were found between microbial turnover rates between groups ($F_{1,3} = 12.6, p < 0.001$). Deer mice in the C-C ($p < 0.001$), C-W ($p < 0.001$) and W-C ($p < 0.001$) groups experienced significantly higher turnover rates than W-W individuals between initial and post-translocation sampling periods. Groups retained the majority (i.e. $>50\%$) of their GMC between captures (C-C = 55.4%; W-C = 55.7%; W-W = 54%), except for the C-W group, which only retained 48.2% of their GMC upon entering wild.

1.4.2 β -diversity and community composition: comparison of gut microbiome communities between wild and captive individuals (initial capture)

PERMANOVA analysis using β -diversity measurements Bray-Curtis (adonis: $F_{1,68} = 5.36, R^2 = 0.15, p < 0.001$) [**Supplementary Figure 1-1A**], Unweighted UniFrac (adonis: $F_{1,68} = 3.82, R^2 = 0.13, p < 0.001$ [**Supplementary Figure 1-2A**]), and Weighted UniFrac (adonis: $F_{1,68} = 8.39, R^2 = 0.25, p < 0.001$ [**Figure 1-6**]), identified significant differences in the GMCs of captive and wild born individuals. Principle co-ordinate analysis ordination modelling identified distinguished clusters of GMCs based on an individual's whether or not an individual was born in captivity or the wild (initial sampling period), with the Weighted UniFrac PCoA model explaining the largest amount of variance (Axis 1: 44.1%, Axis 2: 14.4%).

GMCs of individuals born in the wild contained microbes from 13 different phyla, however, only three phyla (Firmicutes, Bacteroidetes and Proteobacteria) made up at least 1% of the GMC, suggesting that the majority of phyla present are rare. GMCs from individuals born in captivity contained only 10 different phyla, with four phyla (Firmicutes, Bacteroidetes, Proteobacteria and TM7) making contributions greater than 1% of the total abundance (**Figure 1-7**). Significant differences among dominant phyla existed between wild and captive individuals: Firmicutes (mean \pm standard deviation; $68.39 \pm 1.09\%$ vs. $41.13 \pm 1.29\%$, $p < 0.001$), Bacteroidetes ($20.72 \pm 0.53\%$ vs. $50.22 \pm 1.49\%$, $p < 0.001$), Proteobacteria ($8.53 \pm 2.29\%$ vs. $5.50 \pm 4.21\%$, $p < 0.001$) and TM7 ($0.09 \pm 0.21\%$ vs. $1.98 \pm 6.22\%$, $p < 0.001$) (**Supplementary Table 1**).

At the Family level *Ruminococcaceae*, *S24-7* (Phylum: Bacteroidetes), *Lachnospiraceae*, *Lactobacillaceae* and *Helicobacteriaceae* (ϵ -proteobacteria) dominated the gut microbiome of all individuals. *Ruminococcaceae* ($p < 0.001$), *Lachnospiraceae* ($p < 0.01$), and *Helicobacteraceae* ($p < 0.01$) were more abundant in wild individuals, while *S24-7* ($p < 0.001$) and *Enterobacteriaceae* ($p < 0.05$) were more prevalent in captive individuals. *Lactobacillaceae* ($p = 0.98$) showed no significant differences between groups (**Figure 1-8**). Results from the LEfSe analysis complemented these results indicating that when effect size is taken into account these results are still considered significant (**Figure 1-9**).

Constrained analysis of principle components (CAP) identified different patterns in GMCs between wild- and captive-born individuals in terms of which OTUs were enterotype-defining (**Figure 1-10**). Enterotype-defining OTUs had a relative mean abundance $\geq 1\%$ within at least one of the experimental groups, during either the pre- or post-translocation sampling period (**Supplementary Figure 1-3**). The CAP analysis determined the top ten OTUs which were the most influential in differentiating between wild and captive GMCs, by associating different

‘species’ (i.e. OTUs) with each ‘site’ (i.e. captivity or the wild). Eight of the top ten enterotype-defining OTUs for GMCs of wild individuals were identified as Firmicutes including: *Clostridiales* (7, two of which were *Oscillospira* [Family: *Ruminococcaceae*]), *Lactobacillus murinus*, and *Helicobacteraceae* (2) spp. (*Helicobacter rodentium* and *Helicobacter aurati*). Enterotype-defining OTUs for GMCs of captive individuals were made up of *S24-7* (5), *Clostridiales* (2), *Bacteroidales* (2), and *F16* (1) spp. Further identification for different OTUs is presented in **Figure 1-11**, where OTUs are referenced against known microbial species. The *S24-7* microbial family contained a number of OTUs, which were characteristic of GMCs in captive deer mice, whereas *Helicobacteraceae*, and *Ruminococcaceae* microbes were only found in the GMC of wild deer mice. A number of OTUs identified as belonging to the *Lachnospiraceae* family were present in the GMC of both captive and wild deer mice, however no clear taxonomic clustering was apparent.

1.4.3 β -diversity and community composition: post-translocation comparison of gut microbiome communities between groups

After the two-week translocation period, PERMANOVA analysis determined that the GMCs of deer mice who shared the same environment (C-C and W-C; W-W and C-W) showed significant clustering (Bray-Curtis: $F_{1,33} = 4.81$, $R^2 = 0.13$, $p < 0.001$ [**Supplementary Figure 1-1B**]; Unweighted UniFrac: $F_{1,33} = 2.68$, $R^2 = 0.07$, $p < 0.001$ [**Supplementary Figure 1-2B**]; Weighted UniFrac: $F_{1,33} = 3.20$, $R^2 = 0.10$, $p < 0.01$; **Figure 1-12**). The principle component analysis obtained using the Weighted UniFrac PCoA model explained the most amount of variance (Axis 1: 35.9%, Axis 2: 20.6%), however did show a large amount of overlapping between the four experimental groups. Individuals from the two control groups (C-C/W-W) had smaller levels of intra-group heterogeneity, suggesting more homogenous GMCs within these groups.

At the phylum level C-W deer mice had a significantly higher mean relative abundance of Firmicutes compared to W-C deer mice (**Supplementary Table 1-2**). Differences at the family level post-translocation (**Figure 1-13**) showed, *Ruminococcaceae* having higher relative abundance in W-W individuals compared to the C-C ($p < 0.01$) and W-C ($p < 0.01$). *Rikenellaceae* had lower abundances in C-W individuals compared to the W-C ($p < 0.05$) and W-W ($p = 0.052$) groups. Marginal differences were seen when looking at families S24-7 ($X^2 = 6.74$, $p = 0.081$), which was higher in post-translocation captive groups (C-C and W-C) and *Helicobacteraceae* ($X^2 = 6.45$, $p = 0.092$), which was lowest among C-C individuals.

Five OTUs identified in the wild-enterotype were recognized in both initial and post-translocation stage including OTUs identified as *Oscillospira* (2), *Lachnospiraceae* (1) *Helicobacteraceae* (1) and *Lactobacillus murinus* (1). The remaining five OTUs identified for the wild-enterotype post-translocation belonged to *Ruminococcaceae* or *Oscillospira* (3) or within *Clostridiales* (2), suggesting that these particular OTUs have a strong influence and relatively high abundances in GMC composition of mice in natural environments. The captive enterotype recognized only two OTUs between capture periods, which belonged to the microbial families: S24-7 and F16 (phylum: TM7). Other captive enterotype OTUs during the post-translocation stage were identified as *Clostridiales*, *Lachnospiraceae* and *Lactobacillus intestinalis*.

Ruminococcaceae (OTU 51 and OTU 19), *Helicobacteraceae* (OTU 6), *Lactobacillus murinus* (OTU 1), and a *Lachnospiraceae* (OTU 256) spp., strongly characterized the GMC of mice in the wild post-translocation, while S24-7 (OTU 9 and OTU 14), *Lactobacillus intestinalis* (OTU 2) and two *Firmicutes* (OTU 11 and OTU 7) spp., were strongly characteristic for captive deer mice post-translocation (**Figure 1-14**).

1.4.4 β -diversity and community composition: post-translocation comparison of gut microbiome communities within groups

Captive-captive group

Individuals in the C-C group had a marginally significant increase in Firmicutes between capture periods ($p = 0.08$). At the family level a significant increase was seen in *Desulfovibrionaceae* ($p < 0.05$). A marginal increase was seen in *Lachnospiraceae* ($p = 0.059$) as well as decreases in *Lactobacillaceae* ($p = 0.059$) (**Supplementary Figure 1-4**).

Between capture periods C-C individuals experienced increases among all five OTUs identified as *Clostridiales* (**Supplementary Figure 1-5**). *Clostridiales* classified OTUs, which showed significant or marginal changes for C-C were identified as being *Clostridiaceae* (OTU 11, $p < 0.05$; OTU 39, $p = 0.059$; OTU 36, $p = 0.08$) or *Lachnospiraceae* (OTU 21, $p < 0.05$; OTU 7, $p = 0.08$). Significant decreases between capture periods were seen in *Lactobacillus murinus* (OTU 1, $p < 0.05$) and a *S24-7* spp. (OTU 37, $p < 0.05$).

Captive-wild group

C-W individuals experienced consistent changes among Firmicutes (increase, $p = 0.059$), Bacteroidetes (decrease, $p = 0.059$) and Proteobacteria (increase, $p = 0.059$) between capture periods. Although the sample size is small in this group ($n = 5$), changes in the relative abundance of phyla were similar among all individuals, suggesting the marginally significant results are important and provide evidence for biological meaningful relationships. Strong relationships were seen among *Helicobacteraceae* (increase, $p = 0.059$), *Rikenellaceae* (decrease, $p = 0.059$) and *S24-7* (decrease, $p = 0.059$) families upon translocation to the wild (**Supplementary Figure 1-6**). *Ruminococcaceae* ($p = 0.11$) also experienced a strong increasing trend but it was not found to be significant, albeit still considered important due to the level of increase in relative abundance that occurred in all but one individual. LEfSe analysis complemented these findings and identified a

significant increase in *Ruminococcaceae* and decreases in *S24-7* and *Rikenellaceae*. *Helicobacteraceae* was not identified as having a significant impact in differentiating between the GMC of C-W deer mice during the initial and post-translocation capture periods when using the LEfSe analysis, however there was a significant increase in Proteobacteria (**Figure 1-15**).

Similar results were seen at the OTU level, post-translocation C-W individuals experienced a unanimous increase among OTUs that were identified as *Helicobacteraceae* (2) and *Clostridiales* (10). Four OTUs identified as *Clostridiales* could be further identified as *Ruminococcaceae*, the rest belonged to *Clostridiaceae* or *Lachnospiraceae*. Decreases were seen among *Lactobacillus* and *S24-7* groups (**Supplementary Figure 1-7**). All relationships observed for C-W individuals reported a *p*-value of 0.059 suggesting that the small sample size for this group is limiting statistical power.

Wild-Captive group

No significant changes were seen at the Phylum level post-translocation for individuals in the W-C group. At the family level significant decreases were seen in *Helicobacteraceae* ($p < 0.05$) and *Ruminococcaceae* ($p < 0.05$), alternatively *Prevotellaceae* ($p < 0.05$) increased in relative abundance (**Supplementary Figure 1-8**). *Helicobacteraceae* and *Ruminococcaceae* in W-C displayed opposite results compared to individuals from C-W, suggesting they might be tightly linked to extrinsic environmental factors. LEfSe identified *Ruminococcaceae* and *Helicobacteraceae* as being microbial families that significantly decreased between the initial and post-translocation of W-C deer mice and being influential in differentiating GMCs between the two capture periods. Interestingly, the LEfSe analysis failed to identify any microbes that increased during the translocation period in the GMCs of these deer mice that were useful in differentiating GMCs between capture periods.

Opposite to C-W, W-C individuals displayed significant or marginal decreases in all OTUs identified as *Helicobacteraceae* (1, OTU 6 [*Helicobacter rodentium*], $p = 0.052$) and *Clostridiales* (8), except for OTU 26. For the eight OTUs identified as *Clostridiales* that decreased, three of them were classified as *Ruminococcaceae* (OTU 13, $p < 0.05$; OTU 185, $p < 0.05$; OTU 40, $p < 0.05$), while the remaining were located within *Clostridiales* (OTU 60 [*Lachnospiraceae*], $p < 0.05$; OTU 92 [*Lachnospiraceae*], $p < 0.05$; OTU 256 [*Clostridiaceae*], $p < 0.05$; OTU 622 [*Lachnospiraceae*], $p < 0.05$; OTU 641 [*Lachnospiraceae*], $p = 0.052$) (**Supplementary Figure 1-9**). From the *Clostridiales* which displayed reciprocal results between translocated groups, three (OTU 92, OTU 622 and OTU 641) were identified as *Lachnospiraceae*.

Wild-wild group

W-W group individuals did not see any significant changes at the Phylum level. *Rikenellaceae* significantly increased between initial and post-translocation, while *Ruminococcaceae* showed a marginal increase (**Supplementary Figure 1-10**). At the OTU level only OTU 26 (*Eubacterium xylanophilum*, $p = 0.092$) showed any marginal change, decreasing slightly between capture periods (**Supplementary Figure 1-11**).

1.4.5 Maternal influences

During the initial capture phase GMCs were more similar between littermates compared to offspring from other litters in captive born deer mice (Weighted UniFrac: $F_{1.68} = 2.32$, $R^2 = 0.18$, $p < 0.001$). Post-translocation the GMCs of captive born littermates that were translocated to the wild did not cluster with their littermates that remained in captivity (Weighted UniFrac measurements: $F_{1.33} = 0.46$, $R^2 = 0.05$, $p = 0.96$), but instead were more similar to deer mice in the wild (**Figure 16**). Due to low samples sizes there was not enough statistical power to test for significant clustering between siblings who remained in the captivity or between siblings who had

been translocated to the wild, however siblings did not appear to cluster together in captivity or the wild post-translocation.

1.5 Discussion

Results from this experiment provided evidence supporting the hypothesis that the α -diversity and composition of deer mice GMCs were heavily influenced and shaped by an individual's surrounding environment. Neighbouring individuals in the same environment had more similar GMCs and upon being translocated deer mice GMCs homogenized with those of neighbouring deer mice in terms of both α -diversity and GMC composition.

1.5.1 α -diversity

Captivity was associated with lower α -diversity scores relative to wild deer mice, supporting the hypothesis that extrinsic environmental factors influence GMCs. During the initial and post-translocation sampling periods, deer mice in the wild had more diverse GMCs. Clear changes were seen when using Chao1 and Fisher's α -diversity indices, with mostly similar results occurring when using Shannon's diversity index. These results suggest that in captivity, species richness (i.e. Chao1 and Fisher's indices) may be more heavily influenced by changes in the external environment than overall diversity (i.e. Shannon's index - combined richness and evenness), possibly due to a larger number of rare transient microbes acquired from the natural environment. Despite an increase in α -diversity upon being introduced to the wild, C-W individuals failed to achieve the same level of α -diversity as W-W mice. Further studies are needed to determine whether this was caused by the limited time frame of this study (i.e. only a two-week translocation period), or if a lack of exposure to the natural environment limits an individual's ability to achieve the same level of α -diversity as an individual born in the wild.

Inter-individual changes showed that individuals translocated into captivity experienced a significant decreases in α -diversity, while individuals reciprocally translocated to the wild experienced an increase, albeit not significant. Chao1, Fisher's and Shannon's indices all displayed significant or marginal decreases in α -diversity among W-C deer mice between the initial and post-translocation sampling period. These results suggest individuals translocated from the wild into captivity may experience decreases in species richness as well as overall diversity (combined richness and evenness). Although C-W individuals displayed varying differences depending on which α -diversity metric was used, the increase in α -diversity was approximately the same as the decrease seen in W-C individuals. Suggesting that a lack of significance may be due to a lack of statistical power. The increase in α -diversity in C-C individuals (a result from switching from a maternal to solid food based diet) may have made it difficult to see a clear change in C-W individuals post-translocation. Alternatively, these results suggest that translocation from an environment that contains a highly diverse microbial community (i.e. wild environment) to a presumably less-diverse captive environment, has a greater influence on gut microbiome communities than, being reciprocally translocated. Future microbiome studies should be cautious towards interpreting results obtained from wild animals that are temporarily held in captivity.

Increases in microbes among wild individuals may be attributed to a larger available microbial pool (e.g. soil, more diverse diet, greater host richness) than captive individuals (Wang et al. 2014, Adair and Douglas 2017). The results from this study complement previous studies comparing captive to wild populations (Villers et al. 2008, Xenoulis et al. 2010, Nakamura et al. 2011, Cheng et al. 2015, Adair and Douglas 2017), where captive individuals typically had reduced α -diversity compared to their wild counterparts. Greater diversity in food source is also expected to influence gut microbiome composition as demonstrated in captive and wild chimpanzees, where

captive individuals – similar to this study – were fed commercial based diets (Uenishi et al. 2007). C-C individuals saw changes in their GMCs in association with changes in diet, however, their α -diversity did not experience the same increase as their C-W littermates, suggesting that exposure to a vast array of microorganisms (as found in natural environments) is necessary to maximize gut microbiome α -diversity. Similar results have been demonstrated in wild desert woodrats (*Neotoma albigula* and *Neotoma stephensi*), which failed to restore pre-captive GMC diversity levels, upon being reintroduced to a natural diet (Kohl et al. 2014). Together results from this present study and Kohl et al., (2014) suggest that although important, diet is not solely responsible for determining GMC diversity and that other extrinsic environmental factors (e.g. host-host interaction, acquisition through soil and water) must be influencing GMCs. Conflicting results however have been demonstrated. A study on piglets (*Sus scrofa*) showed that individuals reared outdoor had reduced microbial diversity, compared to individuals raised indoor or in isolated houses (Mulder et al. 2009). Experiments have also demonstrated no change in α -diversity between wild and captive lizards (*Liolaemus parvus*, *Liolaemus ruibali* and *Phymaturus williamsi*) (Kohl et al. 2017). Understanding the reasons for these conflicting results justifies further study of GMCs in wild host populations.

1.5.2 Turnover

Based on turnover rates this study suggests that many rare microorganisms are continually being lost and gained, however turnover rates may be further increased when there are changes to an individual's environment (i.e. translocations). As expected W-W deer mice had the most stable GMC (i.e. lowest turnover), likely because they did not undergo environmental or dietary changes. Deer mice in the C-C group transitioned from a maternal based diet to a solid food diet, which is why the turnover rates are thought to have been unexpectedly high. Translocation groups (C-W/W-

C) had the highest turnover rates due to the increased/decreased (depending on the group) levels of exposure to the natural environment. In this study the retention of microbes in GMCs were lower than have been documented in previous studies (Kohl et al. 2014, 2017), potentially caused by individuals in this experiment being juveniles, whose GMCs are inherently less stable than adults.

1.5.3 β -diversity: Environment x gut microbiome interactions

Deer mice inhabiting the same environment (captivity or the wild) contained similar GMCs, supporting the hypothesis that extrinsic environmental factors play an important role in shaping GMCs, complementing previous studies (Uenishi et al. 2007, Villers et al. 2008, Cheng et al. 2015). GMCs of deer mice in the wild and captivity were both dominated by Firmicutes and Bacteroidetes, similar to previous studies examining GMCs among mammalian species (Ley et al. 2008a), and wild mice (Linnenbrink et al. 2013, Maurice et al. 2015, Weldon et al. 2015). However, the wild enterotype was mainly characterized by Firmicutes (*Ruminococcaceae* and *Lachnospiraceae*) and Proteobacteria (*Helicobacteraceae*), while the captive enterotype was mainly composed of Bacteroidetes (*S24-7* and *Bacteroidetes*) as well as some Firmicutes and a TM7 spp.

Post-translocation GMCs were more similar among individuals who shared the same environment, providing further support that the extrinsic environment heavily influences GMC composition. These changes were expected since individuals sharing the same environment would be acquiring microbes from the same species-pool, leading to reduced GMC variation between deer mice in the same environment (Adair and Douglas 2017). The GMC of individuals translocated from captivity to the wild, displayed more similarity to deer mice inhabiting the same environment than to their siblings who remained in captivity. Similar results have been reported

in a lab-based study where GMCs of house mice (*Mus musculus domesticus*) converged after being placed in captive environments with identical diets (Wang et al. 2014). *Lachnospiraceae*, *Ruminococcaceae*, *Helicobacteraceae*, and *Lactobacillaceae* spp. were recognized as being part of the wild-enterotype, providing evidence that these microbes are influential in determining the composition of GMC of deer mice in natural environments. These families made up ~40% of GMCs belonging to individuals in the wild (post-translocation), making it important to understand factors which may influence their abundance.

1.5.4 Gut microbiome community composition x environment

A number of microbial families and OTUs were shown to be strongly associated with exposure to the natural environment. GMCs of individuals in natural environments were dominant by Firmicutes with both *Ruminococcaceae* and *Helicobacteraceae* families showing a strong connection to exogenous environmental exposure. Additionally, a number of OTUs belonging to *Ruminococcaceae*, *Helicobacteraceae* as well as *Lachnospiraceae* demonstrated a strong connection with exposure to the natural environment. Alternatively, S24-7 was the only family and subsequent OTUs which showed a strong consistent connection to captive environments

Firmicutes

Firmicutes made a significantly higher contribution towards the GMC of individuals in an outdoor environment, similar to findings from previous studies (Mulder et al. 2009). The relative abundance of Firmicutes in individuals translocated to the wild increased, while decreasing in individuals translocated into captivity. C-C individuals experienced an increase in Firmicutes, upon switching to a solid food diet, which may have provided an environment more suitable for Firmicutes, including *Lachnospiraceae* and *Clostridiaceae*. Post-translocation individuals in C-C and C-W groups consumed plant material within their diet, however, different food sources are

likely responsible for different OTU abundances (Mackie et al. 2003, Flint et al. 2008, Biddle et al. 2013). The most compelling observation in Mulder et al's. (2009) study was the high abundance of *Lactobacilli* in outdoor reared individuals, alternatively in our study the most influential differences among Firmicutes were seen in *Ruminococcaceae*, and *Lachnospiraceae* spp. In fact, *Lactobacillaceae* (as a family) showed no significant differences between captive and wild born individuals in this study, possibly due to intra-genus specializations (Schloss et al. 2012). Due to the commonality among *Lactobacillus* and immune-benefits provided, it is possible that some *Lactobacillus* spp., are vertically transmitted to offspring through maternal sources (passage through the birth canal; vaginal microbial community [Funkhouser and Bordenstein 2013]).

Ruminococcaceae and *Lachnospiraceae* spp. demonstrated strong links with extrinsic environmental influences. The relative abundance of *Ruminococcaceae* and *Lachnospiraceae* spp. (OTU 13, 185, 60, 92, 622, and 641) increased among individuals translocated into the wild and decreased among individuals translocated to captivity, demonstrating a reciprocal effect, providing evidence that these OTUs thrived when deer mice were placed within natural environments. Ruminococci have also previously been shown to be predominant in wild chimpanzees compared to captive counterparts (Uenishi et al. 2007), however, conflicting findings have been demonstrate in desert woodrats (*Neotoma lepida*), which increased in *Ruminococcus* (*Ruminococcaceae* genus) and *Coprococcus* (*Lachnospiraceae* genus) upon entering captivity (Kohl and Dearing 2014). Differences seen between desert woodrats and deer mice being brought into captivity, may be caused by both species consuming different diets, which likely promotes different sets of specialized microbes. *Ruminococcaceae* and *Lachnospiraceae* both perform functional roles as degraders of complex plant material, suggesting they play an important role in allowing their hosts to extract nutrients from plant rich-diets (Mackie et al. 2003, Flint et al. 2008, Biddle et al. 2013).

Different diets likely promoted the growth of different species, as seen by both C-C and C-W; individual's GMCs both increased in relative abundance of different OTUs within the *Clostridiales* order. Ecological and evolutionary differences between captive and wild associated *Clostridiales* OTUs should be teased apart within future research, to determine their functional differences.

Lower taxonomic classification identified *Oscillospira* genera as influential microbes within the GMCs of wild individuals, complementing results obtained by McKenzie et al. (2017). *Oscillospira* is an understudied group despite being common in humans as well as murines. Current research available has determined that *Oscillospira* is inversely related to susceptibility of inflammatory disease (i.e. Crohn's disease) (Konikoff and Gophna 2016), and may play an important role in healthy GMCs. Intra-genus specialization occurred within the *Lactobacillus* genus which has been shown to benefit host immune systems in a variety of vertebrates (Fuller 1989, Guarner and Malagelada 2003, Gardiner et al. 2004, Perelmutter et al. 2008, Funkhouser and Bordenstein 2013). *Lactobacillus murinus* was found to be influential in GMC of wild deer mice in particular has been shown to have immune positive affects in both dogs and porcine (Gardiner et al. 2004, Perelmutter et al. 2008). Future research should focus on understanding the potential fitness benefits associated with early life exposure to *Oscillospira*, and *Lactobacillus* spp., to understand their ecological and evolutionary interactions with mammalian hosts.

Bacteroidetes

S24-7 microbes did not appear to be strongly associated with exposure to the natural environment. *S24-7* microorganisms were more abundant in captive individuals similar to findings by McKenzie et al. (2017), however, their presence in wild mice suggests that some species within this family are a naturally occurring members of mice GMCs. Results from this study complement previous studies suggesting that *S24-7* microbes may be associated with a combination of maternal

(Dominguez-Bello et al. 2016) and dietary (Evans et al. 2014, Ormerod et al. 2016) sources. Both C-C and C-W deer mice displayed decreases in OTUs classified as *S24-7*, suggesting a maternal influence. However, post-translocation C-C and W-C displayed higher abundances of *S24-7* compared to C-W and W-W, suggesting that some microbes within this group thrived on the captive-based diet as well. *S24-7* microbes are associated with the breakdown of proteins and carbohydrates (Serino et al. 2012, Evans et al. 2014), and may have been more suitable for the diet deer mice experienced in captivity, during this study. Another possibility is that captive individuals were able to maintain higher relative abundances of *S24-7* compared to C-W and W-W individuals due to decreased microbial exposure, resulting in less competition within GMCs of captive deer mice.

Similar to findings from Kohl et al., (2017) and McKenzie (2017), individuals in captivity (during the initial sampling stage) had relatively high abundances of *Enterobacteriaceae*. Post-translocation *Enterobacteriaceae* decreased among all groups except W-C. In the GMCs of W-C deer mice, *Enterobacteriaceae* showed an increasing trend upon entering captivity. *Enterobacteriaceae* microbes have been shown to represent early colonizers (O'Hara and Shanahan 2006) and maintain a limited presence in GMCs of wild mice (Maurice et al. 2015). Increased presence of *Enterobacteriaceae* in captive individuals should be further examined in future studies, since microbes in this family such as *E.coli* and *Salmonella* can serve as pathogenic indicator species (Gardiner et al. 2004).

Proteobacteria

Helicobacteraceae was strongly linked to exposure to extrinsic environmental factors. The GMCs of deer mice translocated to the wild increased in the relative abundance of *Helicobacteraceae*, while displaying the opposite pattern in deer mice translocated into captivity.

Similar to *Ruminococcaceae* and *Lachnospiraceae* OTUs, *Helicobacteraceae* classified OTUs showed reciprocal relationships in deer mice translocated between environments, increasing abundance in individuals translocated to the wild and decreasing in deer mice translocated to captivity. *Helicobacteraceae* have been identified in the gut microbiome of mice (including *Peromyscus* spp.) previously and are expected to be common within GMCs (Baxter et al. 2015, Maurice et al. 2015, Brooks et al. 2016). *Helicobacteraceae* showed a consistent decrease among W-C individuals, and was lowest in C-C individuals (approximately four times lower than their translocated littermates), providing further evidence that *Helicobacteraceae* species are more prominent in natural environments. This present study, complementary to Baxter et al. (2015), provides evidence that under normal conditions *Helicobacteraceae* spp. display commensal relationships with their hosts and are commonly found within GMCs of mice. However, under co-occurring infection by *H. hepaticus* in immunodeficient mice, *Helicobacter rodentium* was shown to become pathogenic (Myles et al. 2004). Suggesting that mice may potentially be acting as reservoir hosts to pathogenic and zoonotic *Helicobacteraceae* strains (Wasimuddin et al. 2012).

The increased presence of *Desulfovibrionaceae* in captive C-C may have been caused by the captive-chow. Previous studies on lizards (*Liolaemus ruibali*) has demonstrated a correlation between fibre digestibility and *Desulfovibrio* (Kohl et al. 2016), however, it is important to note that no pattern was seen between entering captivity and *Desulfovibrionaceae* in W-C individuals.

1.5.5 Maternal and sex influences

Maternal influences were present during the initial sampling of captive-born deer mice, as the GMCs of deer mice from the same litter clustered closer together than non-related deer mice. However, post-translocation C-W deer mice contained GMCs that were more similar to wild deer mice than their siblings that remained in captivity. Although maternal effects have been

demonstrated in lab-based studies there is growing evidence that in natural populations maternal influences on microbiome communities only occur in early-life stages and are short lasting (Lucas and Heeb 2005, Kovacs et al. 2011, Campbell et al. 2012). Although few whole litters were resampled in the wild, individuals that were translocated to the wild contained GMCs more similar to non-related individuals from the wild rather than their genetically related siblings in captivity.

Only marginal sex differences were seen among individuals born in captivity, likely because individuals were cohabitating and had the same diet. Similarly in the wild only marginal changes were seen, possibly as a result of deer mice being juveniles and not being reproductively mature. Schloss et al. (2012) found similar results in their experiment using C57BL/6 inbred mice, whereby no sex differences were apparent between juvenile mice. Additionally, sex only explain ~5% of variation seen within GMCs for both captive and wild born deer mice in this study, suggesting that although differences may be present, they were not very influential.

1.5.6 Conservation perspectives

GMCs have long been considered to be beneficial for their hosts by collaborating in physiological development (Falk 1998, Heijtz 2001, Cash and Hooper 2005, Round and Mazmanian 2009), and metabolic processes (Hooper 2002), making it important for captivity based conservation programs to consider GMCs within captive populations, especially when considering reintroductions (Redford et al. 2012). Animals translocated from captivity to the wild often have reduced survival and foraging success rates (DeGregorio et al. 2017), possibly as a result of possessing microbiome communities poorly adapted to novel diets they encounter within new environments (McKenzie et al. 2017).

Results from this study suggest that continued exposure to the exogenous environment is important to maintaining diverse GMCs and should be considered when translocating animals

from wild to captive environments. Animals in captivity may not be able to obtain a GMC that is as diverse as GMCs found in wild animals, however, upon being translocated into the wild from captivity, individuals may be able to acquire a highly diverse GMC over time. Therefore the GMCs of captive animals that are translocated to the wild should be able to change to accommodate changes in their surrounding environments. Although, further research should be conducted to determine if translocated individuals are exposed to detrimental effects (e.g. decreased digestion efficiency) which may affect their chances of survival during this GMC transitional stage. Microorganisms associated with natural environments in deer mice used in this study were *Ruminococcaceae*, *Helicobacteraceae* and *Lachnospiraceae*. Increased exposure to these microbial families when in captivity may provide captive animals with GMCs would similar to those of wild animals and could potential limit the transitional phase of their GMCs upon being translocated to the wild. Future research should examine potential fitness consequences associated with a decreased presence of these families in early life stages, to determine if early life exposure of these families would benefit individuals born in captivity.

1.5.7 Conclusion

Determining the influence of extrinsic environmental factors on GMCs is important in helping to understand evolutionary and ecological relationships between microbes and their hosts. Recent studies have ventured beyond the use of germ-free or captive populations in an attempt to understand GMCs in natural populations, however, these studies are still underrepresented in the literature (Hird 2017). This study goes beyond previous work by examining the effects of translocation on GMCs, a topic which has to date received little attention.

Exposure to extrinsic environmental factors was associated with increased α -diversity and community composition changes in GMCs, demonstrating that external sources can have a

profound impact on GMC composition. GMCs quickly changed to match those of individuals in similar environments, exhibiting high turnover rates upon translocation. Despite high turnover rates and changes in GMC composition C-C individuals (which switched to a solid food diet over the course of the experiment), retained lower levels of α -diversity. Evidence from this study suggests that individuals inhabiting natural environments harbour a vast array of microbes compared to their captive counterparts, and that continued exposure to a large microbial species pool is necessary for individuals to maintain diverse GMCs. *Ruminococcaceae*, *Lachnospiraceae*, and *Helicobacteraceae* spp., were all abundant in wild deer mice GMCs and showed reciprocal changes between individuals translocated from captivity to the wild and vice versa, suggesting that these microbes flourish when deer mice are in natural environments. Although common, *S24-7* spp., were not found to be associated with exposure to the natural environment. Maternal influences, although initially present, were quickly masked upon exposure to natural environmental conditions. Sex influences were present among individuals in the wild, but explained only a small amount of variation, suggesting that sex differences have a limited impact on GMCs compared to the combined influence of other extrinsic environmental factors.

1.5.8 Concluding remarks and future directions

Along with considering guidelines set by Goodrich et al. (2014), future studies should consider using this study as a template, when attempting to understand how the exogenous environment may influence GMCs. This study was able to determine which microbial families as well as important OTUs which are associated with natural environments, by examining how GMCs are influenced when individuals are translocated between environments. *Ruminococcaceae*, *Lachnospiraceae* and *Helicobacteraceae* abundances increased upon environmental exposure, but potential fitness consequences due to delayed exposure in life should be further studied to

understand potential fitness consequences. While future studies should continue to examine wild populations, lab-based studies should be used collaboratively (Yi and Li 2012) in determining ecological and evolutionary relationships which exist between microbes correlated with exposure to the natural environment and their mammalian hosts.

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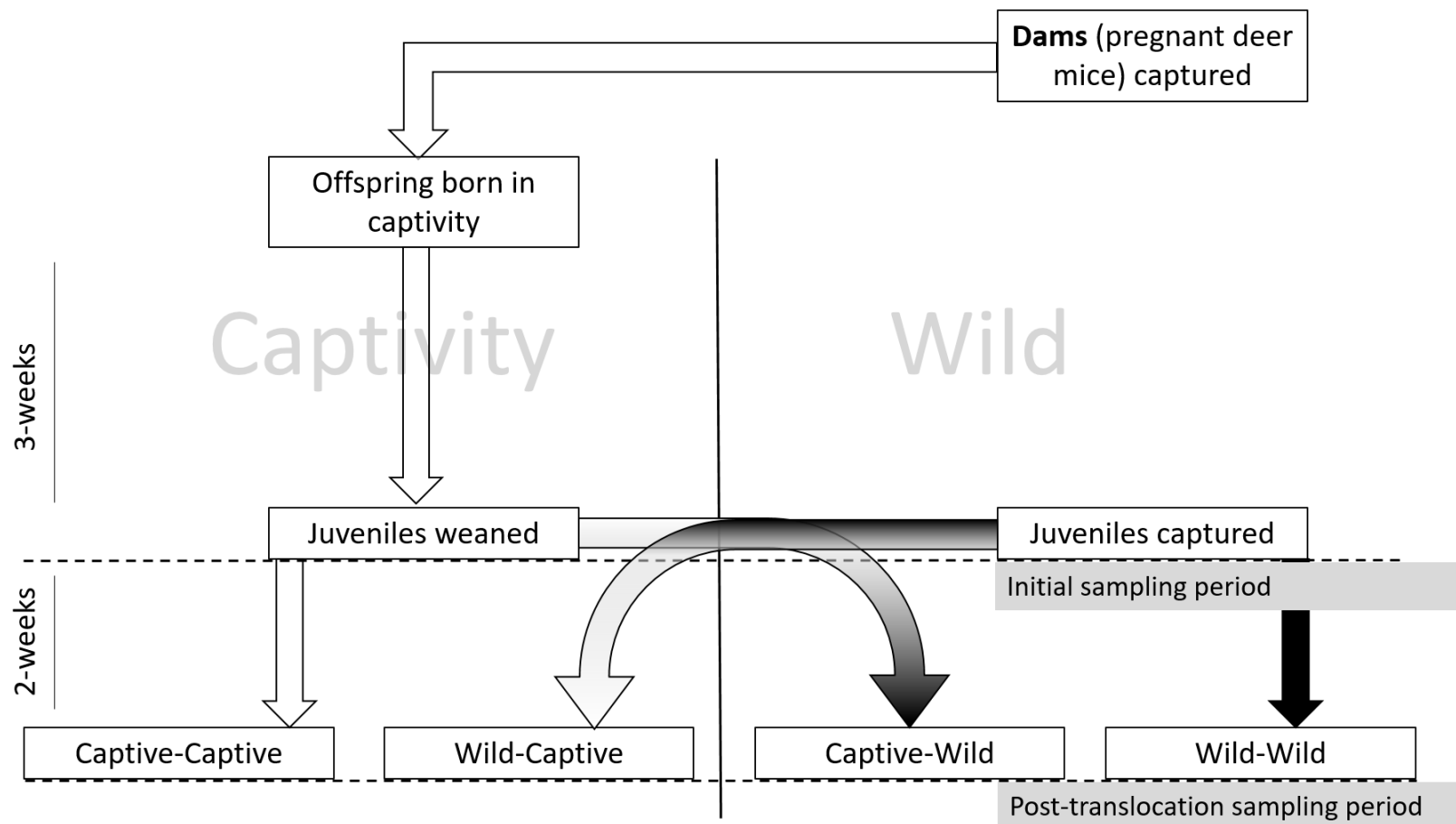


Figure 1-1: Diagram demonstrating the experimental design followed for this study. Pregnant dams were captured in the wild (May-early June) and transferred to the Algonquin Provincial Park Wildlife Research Station so that their offspring would be born in captivity. Three-weeks later after the captive juveniles were born their initial fecal samples were collected. A sub-sample of captive born individuals were then translocated to the wild (Captive-Wild group). Mice that remained in captivity (Captive-Captive group) along with their translocated siblings were then recaptured and sampled again two-weeks (post-translocation sampling period) after the initial sampling period had occurred. Wild juvenile deer mice were sampled in the wild during the initial sampling period, a sub-sample of mice were then translocated to captivity (Wild-Captive group). Two-weeks later mice translocated from the wild to captivity as well as mice that remained in the wild (Wild-Wild group) were sampled again (post-translocation sampling period).

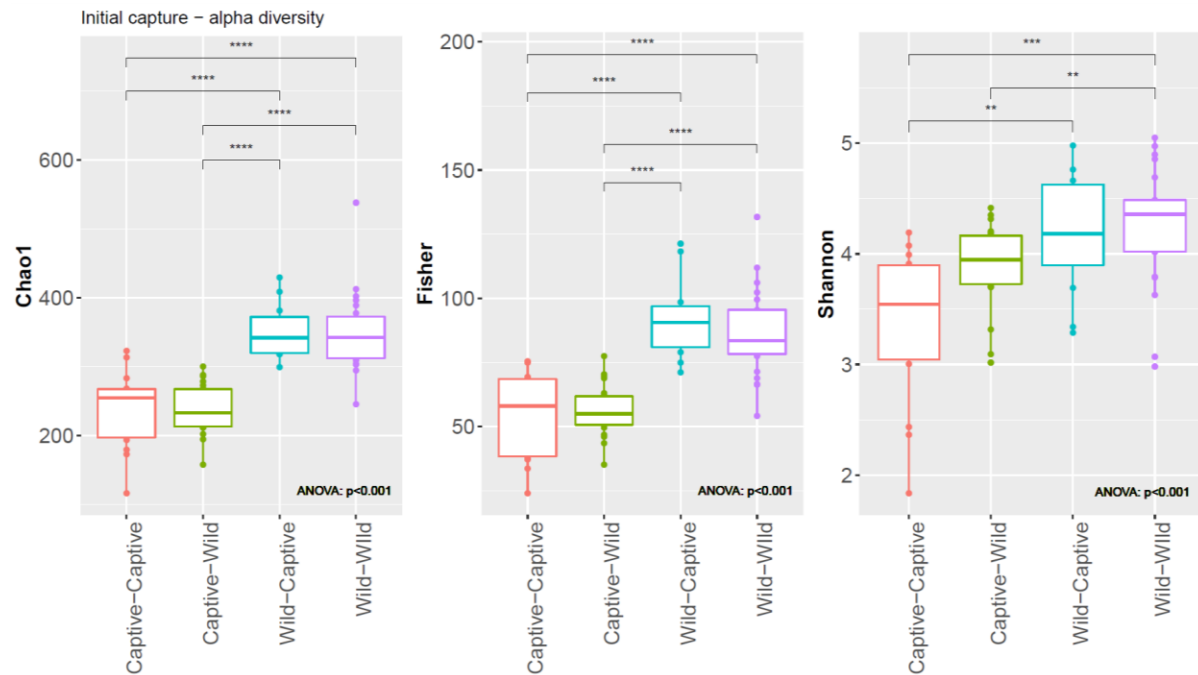


Figure 1-2: Initial capture results show that there were differences in α -diversity levels among the four experimental groups during the initial sampling phase of the experiment. Chao1 ($F_{1,3} = 32.23$, $p < 0.01$) and Fisher α -diversity ($F_{1,3} = 28.07$, $p < 0.01$) indices show significant differences between deer mice born in different environments ($p < 0.01$; for all comparisons among groups where individuals were born in different environments). Shannon's diversity index ($\chi^2_{1,3} = 19.08$, $p < 0.01$) showed similar results as Chao1 and Fisher except there was no significant difference between the Captive-Wild/Wild-Captive groups ($p = 0.11$). **** indicates $p < 0.0001$, *** indicates $p < 0.001$ and ** indicates $p < 0.01$.

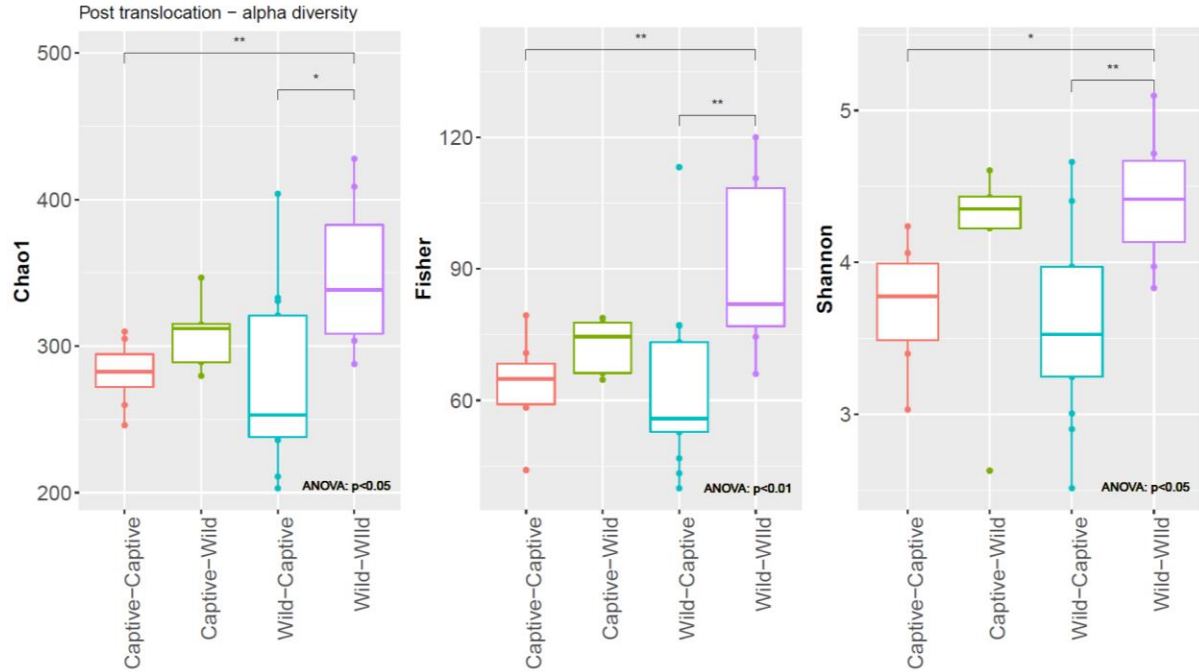


Figure 1-3: Chao1, Fisher and Shannon α -diversity levels displayed marginal or significant difference between experimental groups during the post-translocation sampling period. There was a significant difference between the experimental groups during the post-translocation sampling period when using the Chao1 α -diversity index ($F_{1,3} = 4.08, p < 0.05$). Wild-Wild deer mice had significantly more diverse gut microbiome communities compared to Captive-Captive deer mice ($p = 0.01$), with a marginal difference between Wild-Wild/Wild-Captive deer mice ($p = 0.069$). The Fisher α -diversity scores ($F_{1,3} = 5.074, p < 0.01$) showed significant differences between Wild-Wild/Wild-Captive deer mice ($p < 0.05$) and Wild-Wild/Captive-Captive deer mice ($p < 0.01$). Shannon's index scores ($\chi^2_{1,3} = 10.44, p < 0.05$) showed a significant difference between Wild-Wild/Wild-Captive deer mice ($p < 0.05$) and a marginal difference between the Wild-Wild/Captive-Captive groups ($p = 0.055$). ** indicates $p < 0.05$ and * indicates $p < 0.1$.

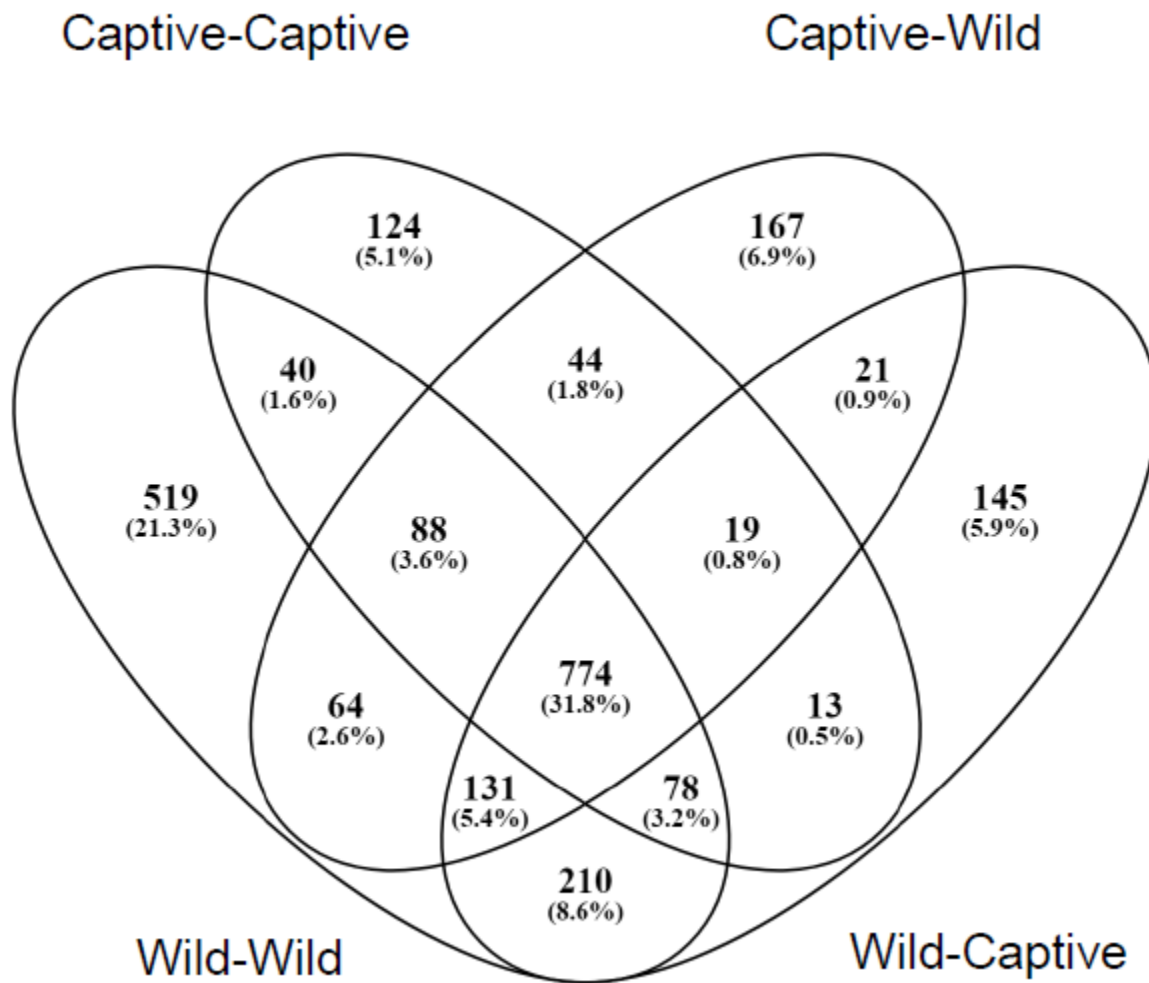


Figure 1-4: Venn diagram displaying all the OTUs identified in deer mice during the post-translocation phase of the experiment. Wild-Wild individuals contained 519 (21.3%) unique OTUs, more than three times the amount of unique OTUs belonging to any of the other experimental groups. A large number of OTUs (i.e. 774 OTUs [31.8%]) were shared between all four experimental groups.

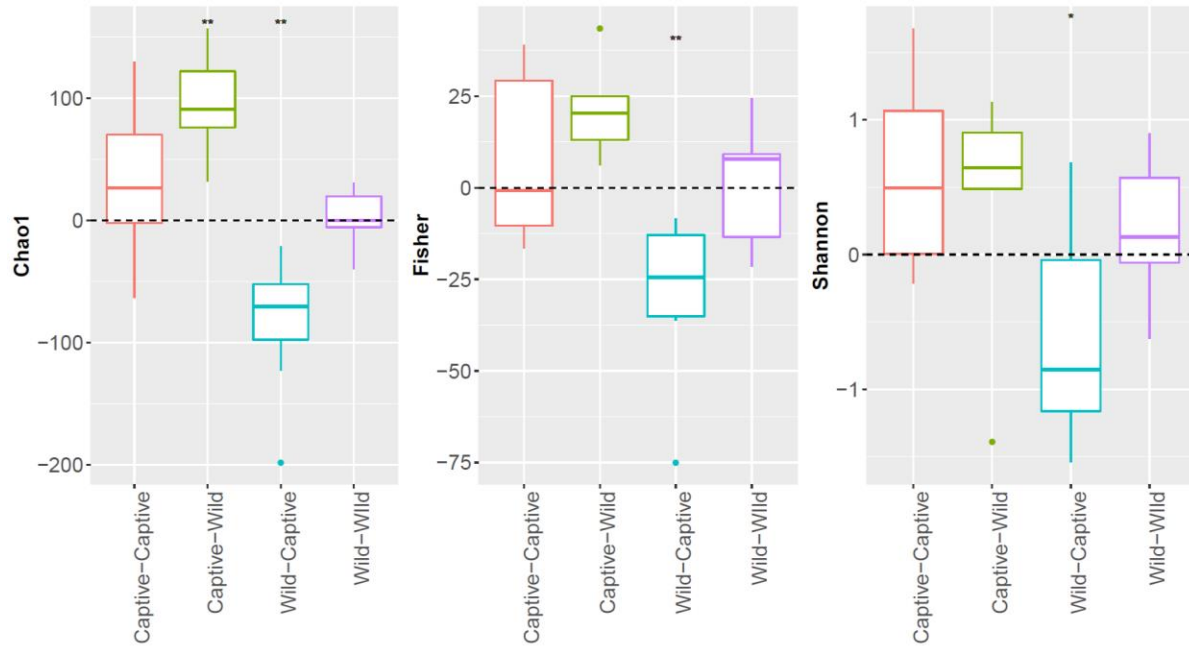


Figure 1-5: Changes in α -diversity levels between initial and post-translocation sampling periods were identified within different groups using different α -diversity indices. Chao1 α -diversity levels showed a significant increase for deer mice in the Captive-Wild group ($p < 0.01$) as well as a significant decrease for deer mice in the reciprocally translocated Wild-Captive group ($p < 0.01$). The only significant change in α -diversity between sampling periods seen when using Fisher's ($p < 0.01$) and Shannon's ($p = 0.08$) indices was a decrease for deer mice in the Wild-Captive group. ** indicates $p < 0.05$ and * indicates $p < 0.10$.

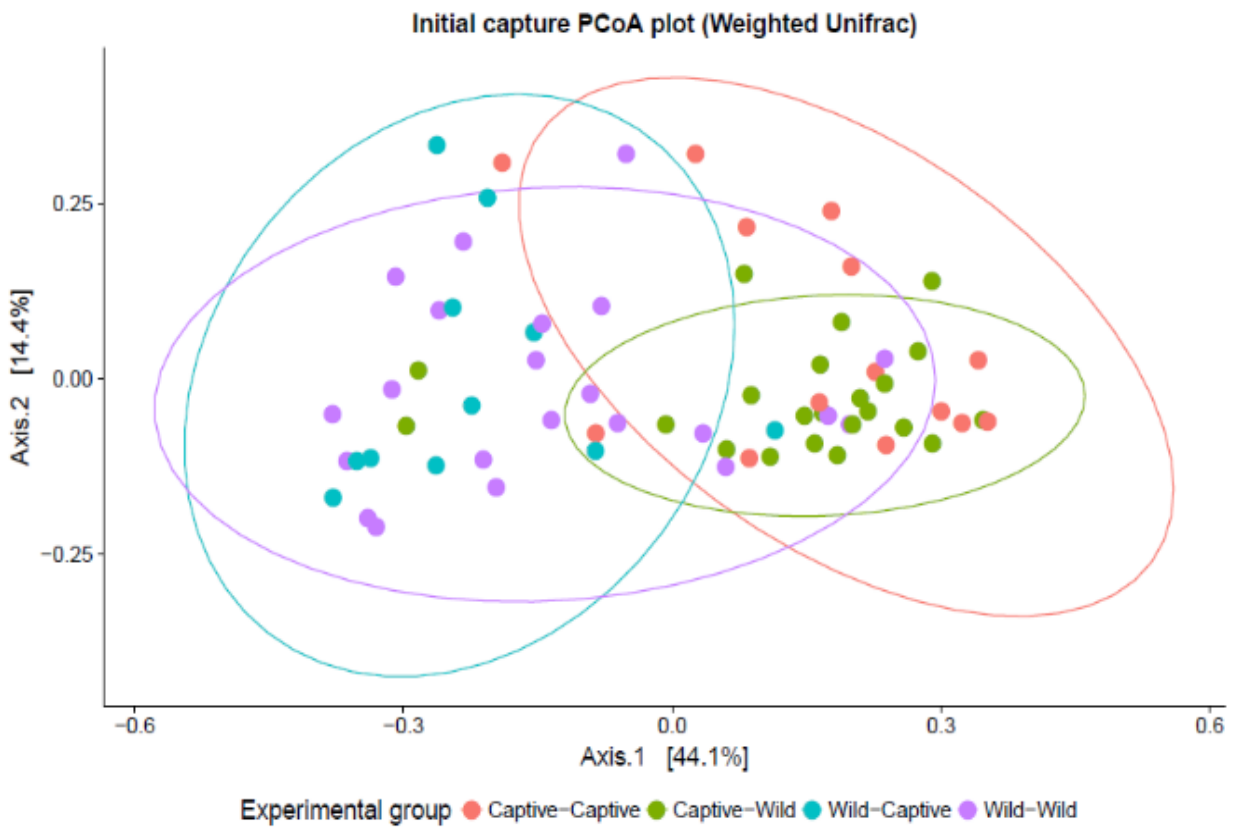


Figure 1-6: Principle coordinate analysis (PCoA) ordinations using weighted UniFrac measurements determined that the gut microbiome communities of deer mice born in the same environment clustered together, during the initial sampling period (adonis: $F_{1,3} = 7.20$, $R^2 = 0.25$, $p < 0.01$).

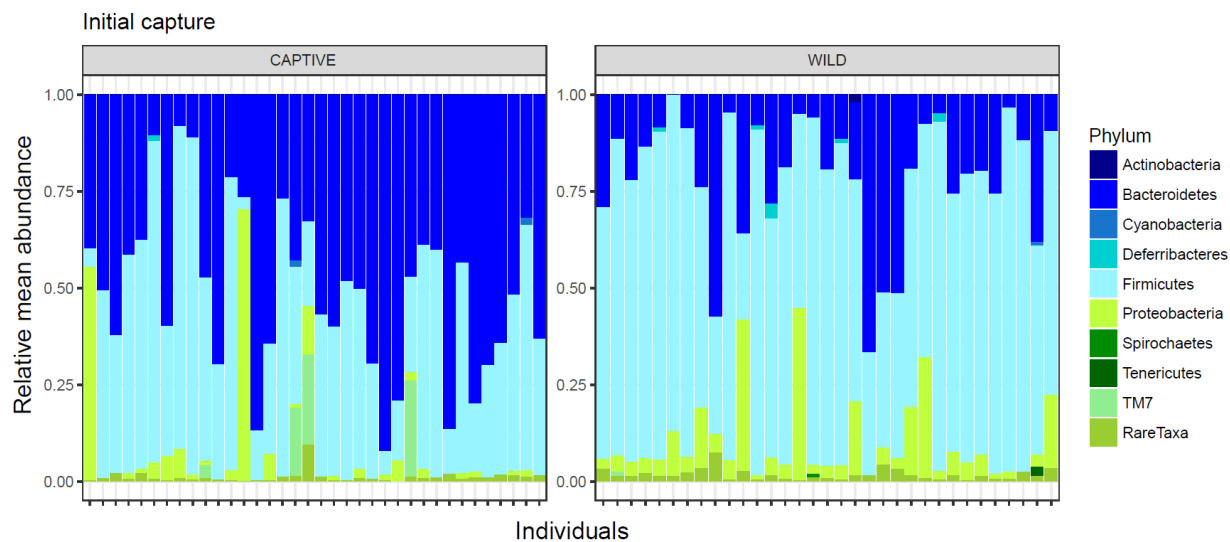


Figure 1-7: The relative abundance of microbes (classified at the phylum level) that compose the gut microbiome communities (GMCs) of deer mice born in the captive and wild environments. The GMCs of deer mice born in captive environments are dominated by Bacteroidetes followed by Firmicutes. The GMCs of deer mice born in wild (natural) environments are dominated by Firmicutes followed by Bacteroidetes, the opposite of captive born deer mice. Proteobacteria also appear to be more consistently abundant in wild born deer mice compared to captive born individuals. Each bar represents the GMC of an individual deer mouse.

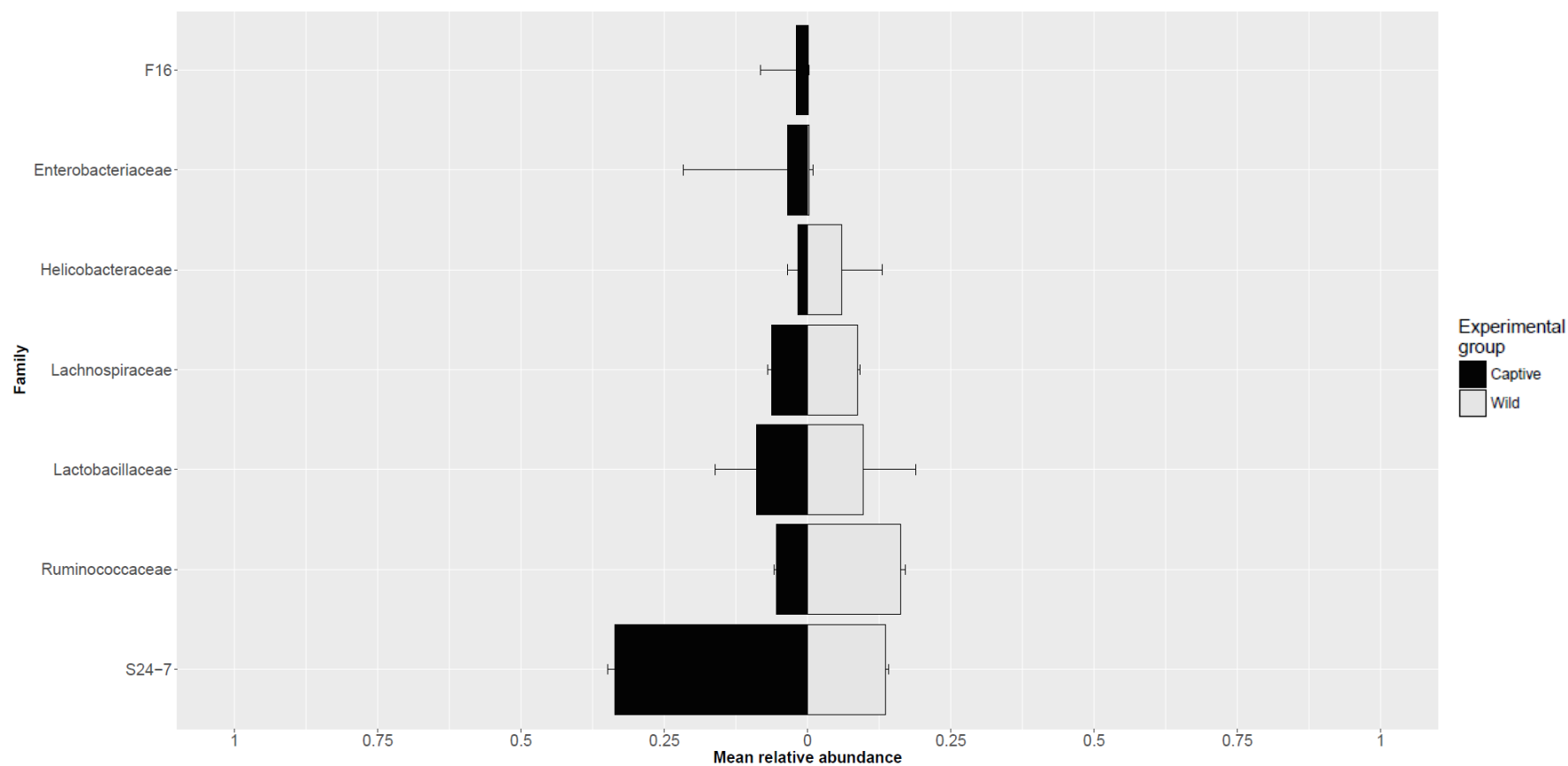


Figure 1-8: Side-by-side comparison of the mean relative abundance of families which made up at least >1% of the total gut microbiome community, within at least one individual, during the initial capture period for deer mice born in captivity and the wild. Significant differences occur between the *Ruminococcaceae* ($p < 0.001$), *Lachnospiraceae* ($p < 0.01$), *Helicobacteraceae* ($p < 0.01$), *S24-7* ($p < 0.001$) and *Enterobacteriaceae* ($p < 0.05$)†. *Lactobacillus* showed no significant difference between captive and wild groups during the initial sampling period. †This relationship seemed to be driven by just two individuals, see *Supplementary Figure 1-3*.

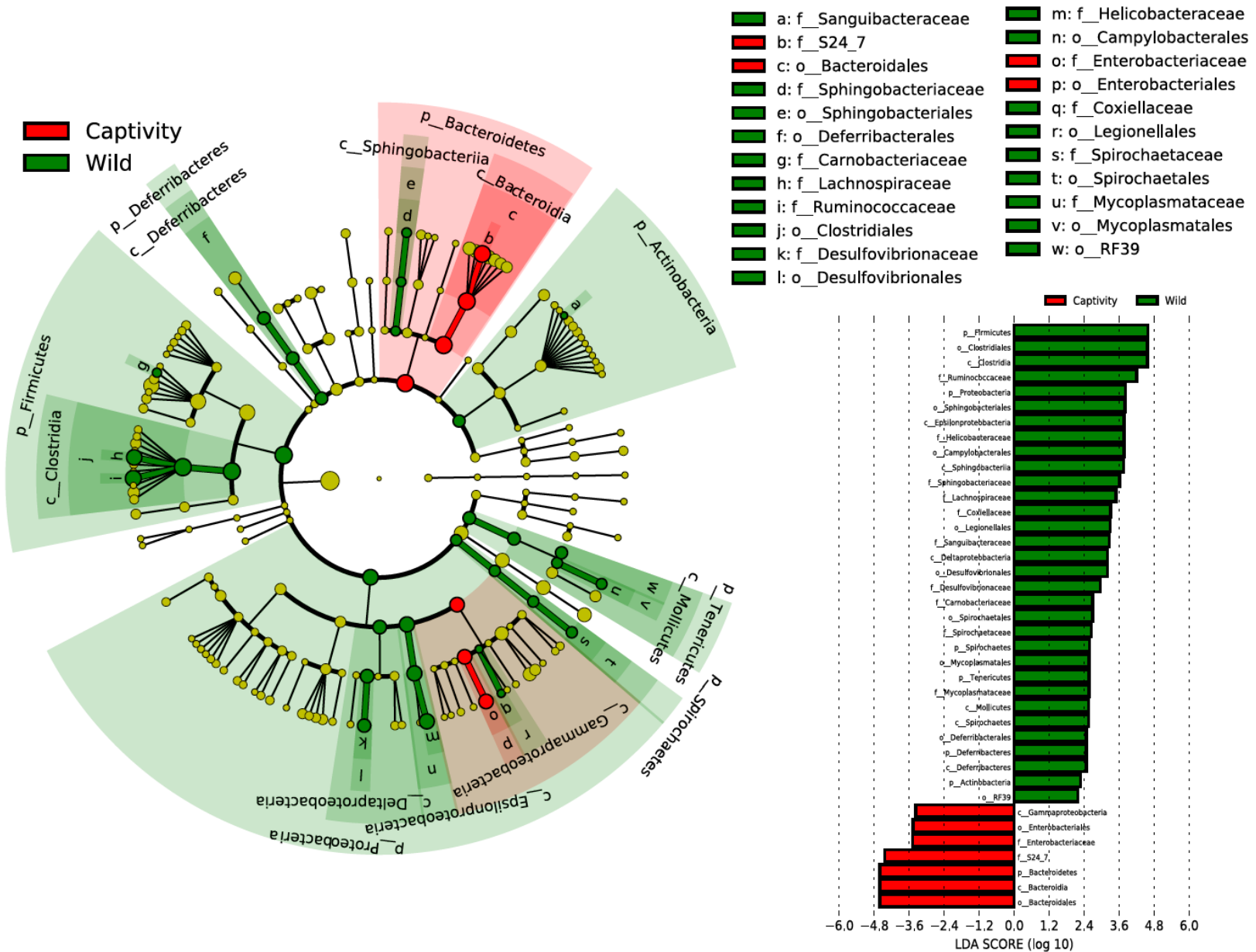


Figure 1-9: Linear discriminant analysis (LDA) effect size (LefSe) determined that there were a number of microbial families that were more abundant in the GMCs of wild deer mice compared to GMCs of captive raised deer mice (initial sampling period), including *Ruminococcaceae*, *Helicobacteraceae*, and *Lachnospiraceae*, which composed $\geq 1\%$ of gut microbiome communities in deer mice. Alternatively, microbial families S24-7 and *Enterobacteriaceae* (composed $\geq 1\%$ of gut microbiome communities in some captive deer mice individuals) were found to be significantly more abundant in captive deer mice.

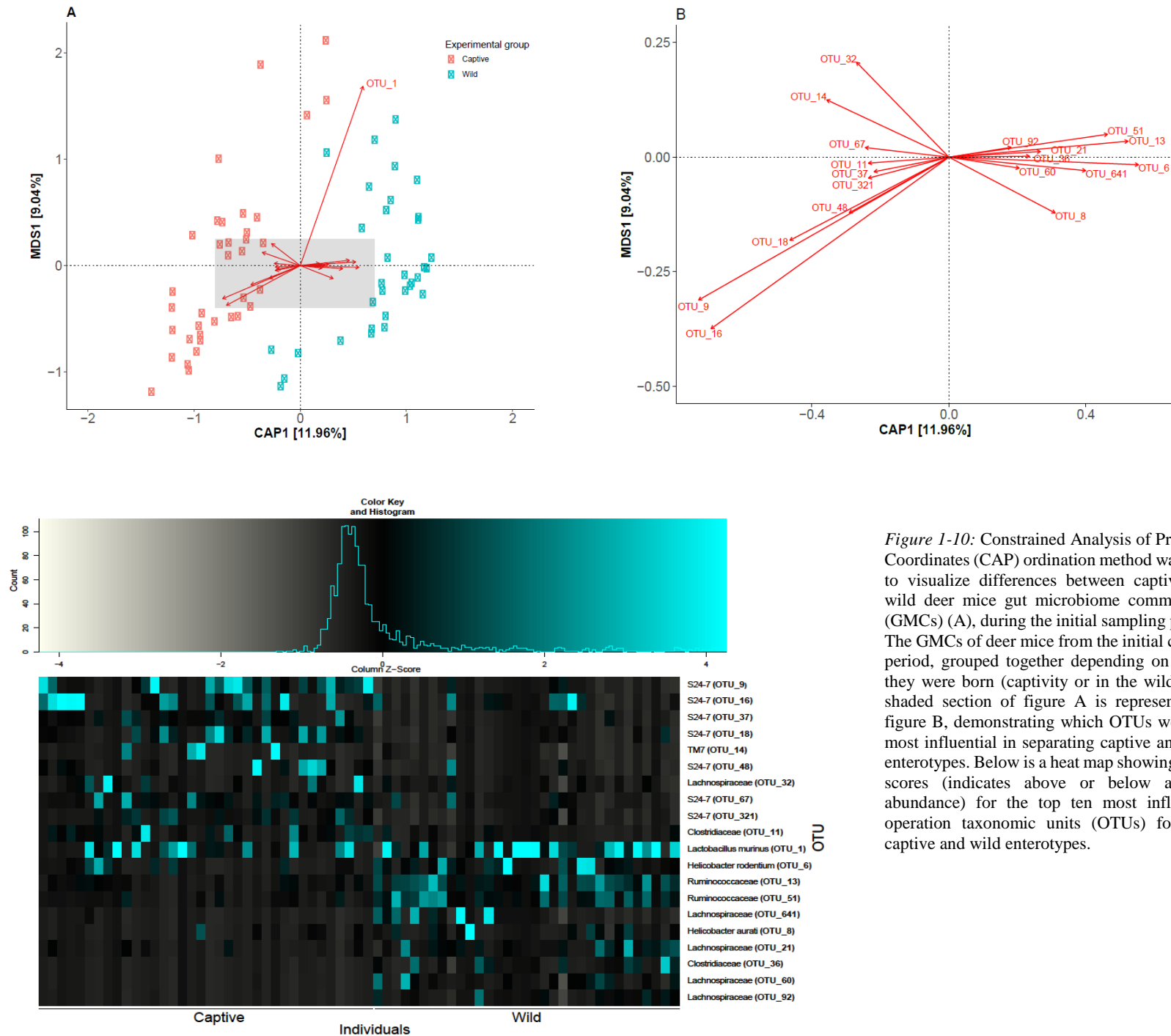


Figure 1-10: Constrained Analysis of Principle Coordinates (CAP) ordination method was used to visualize differences between captive and wild deer mice gut microbiome communities (GMCs) (A), during the initial sampling period. The GMCs of deer mice from the initial capture period, grouped together depending on where they were born (captive or in the wild). The shaded section of figure A is represented in figure B, demonstrating which OTUs were the most influential in separating captive and wild enterotypes. Below is a heat map showing the z-scores (indicates above or below average abundance) for the top ten most influential operation taxonomic units (OTUs) for both captive and wild enterotypes.

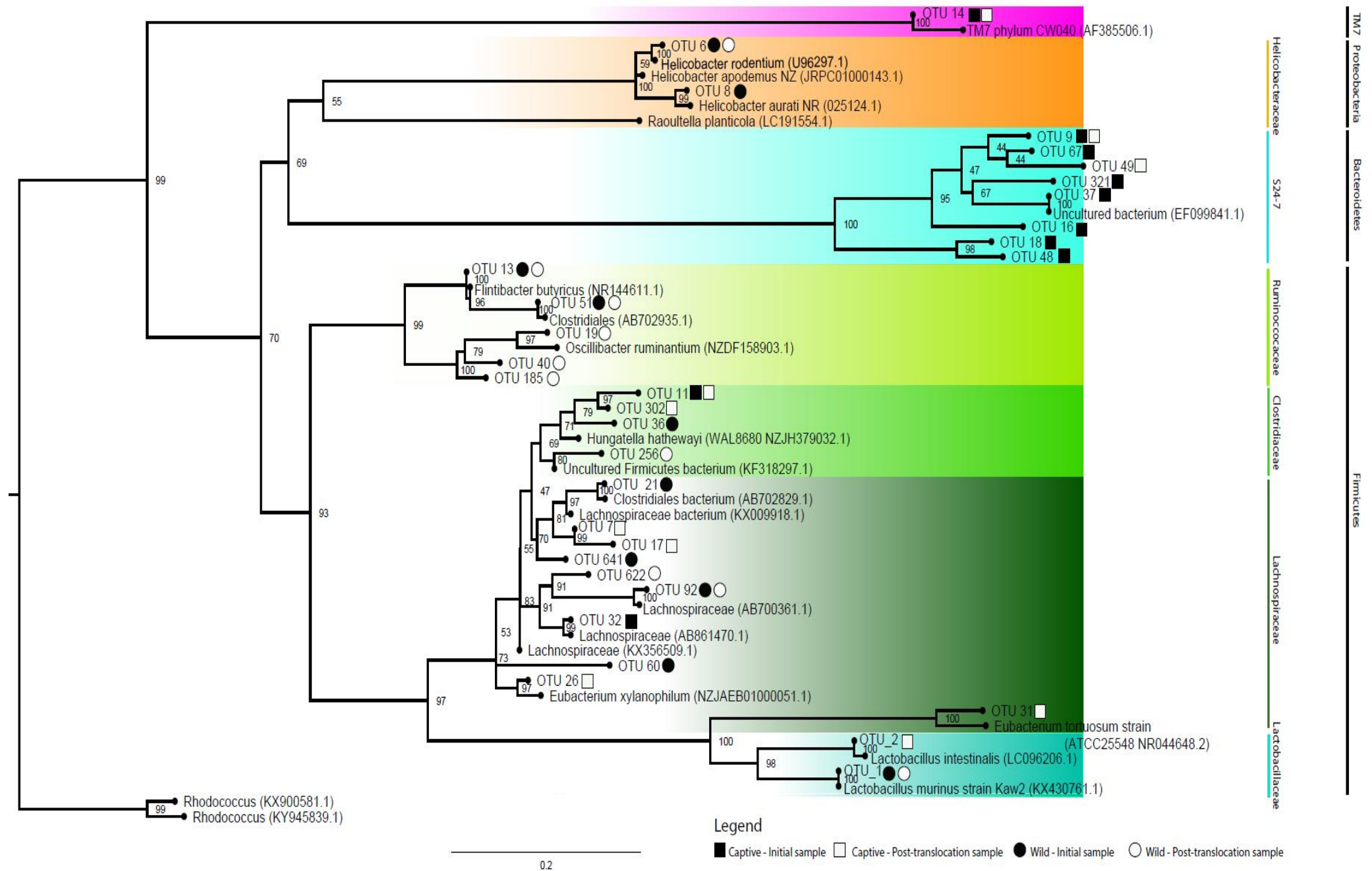


Figure 1-11: Phylogenetic tree containing the top ten most influential Operational Taxonomic Units (OTUs) for captive and wild enterotypes during initial and post-translocation sampling periods. Black squares indicate that an OTU was characteristic for the captive enterotype during the initial capture stage, while white squares indicate that an OTU was characteristic among gut microbiome communities for deer mice in captivity during the post-translocation sampling period. Black circles indicate that an OTU was important for the wild enterotype during the initial capture stage, while white squares indicate that an OTU was important among gut microbiome communities for deer mice in wild during the post-translocation sampling period. A number of *S24-7* microbes were clearly associated with captivity, while *Helicobacteraceae* and *Ruminococcaceae* microbes were associated with the wild environment. *Lachnospiraceae* microbes did not show any clear clustering. Inter-genus specializations occurred within the *Lactobacillaceae* family.

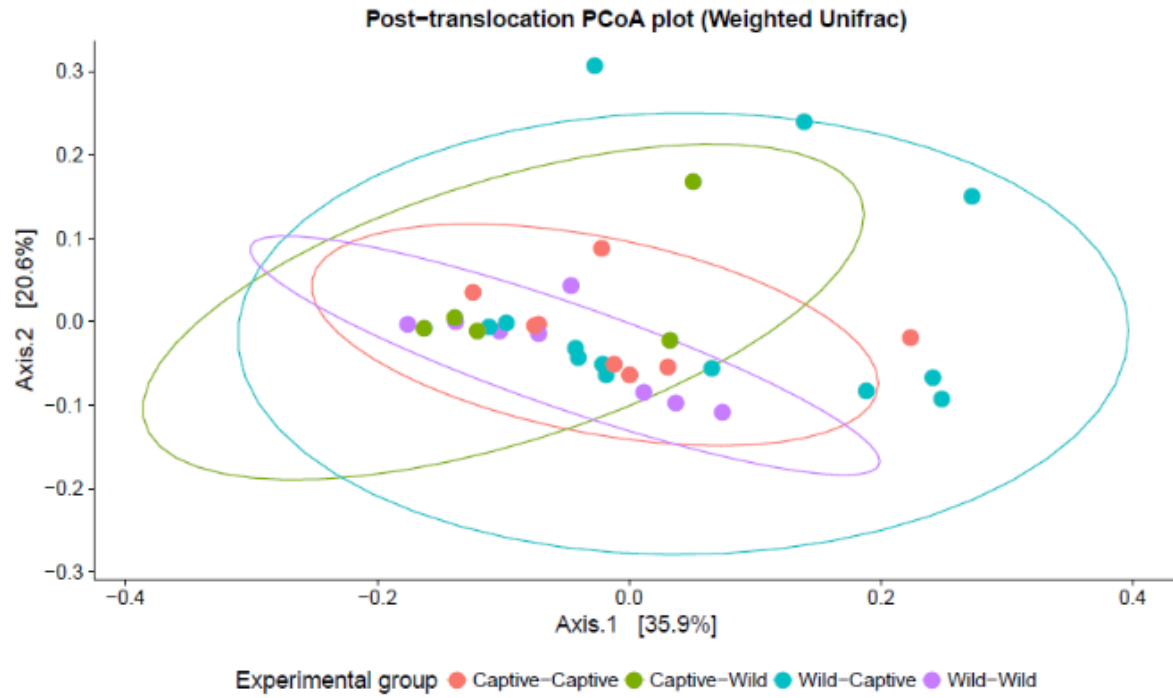


Figure 1-12: Principle coordinate analysis (PCoA) ordinations using weighted UniFrac measurements determined that the gut microbiome communities of neighbouring deer mice in the same environment post-translocation clustered together (adonis: $F_{1,33} = 3.20$, $R^2 = 0.10$, $p < 0.01$).

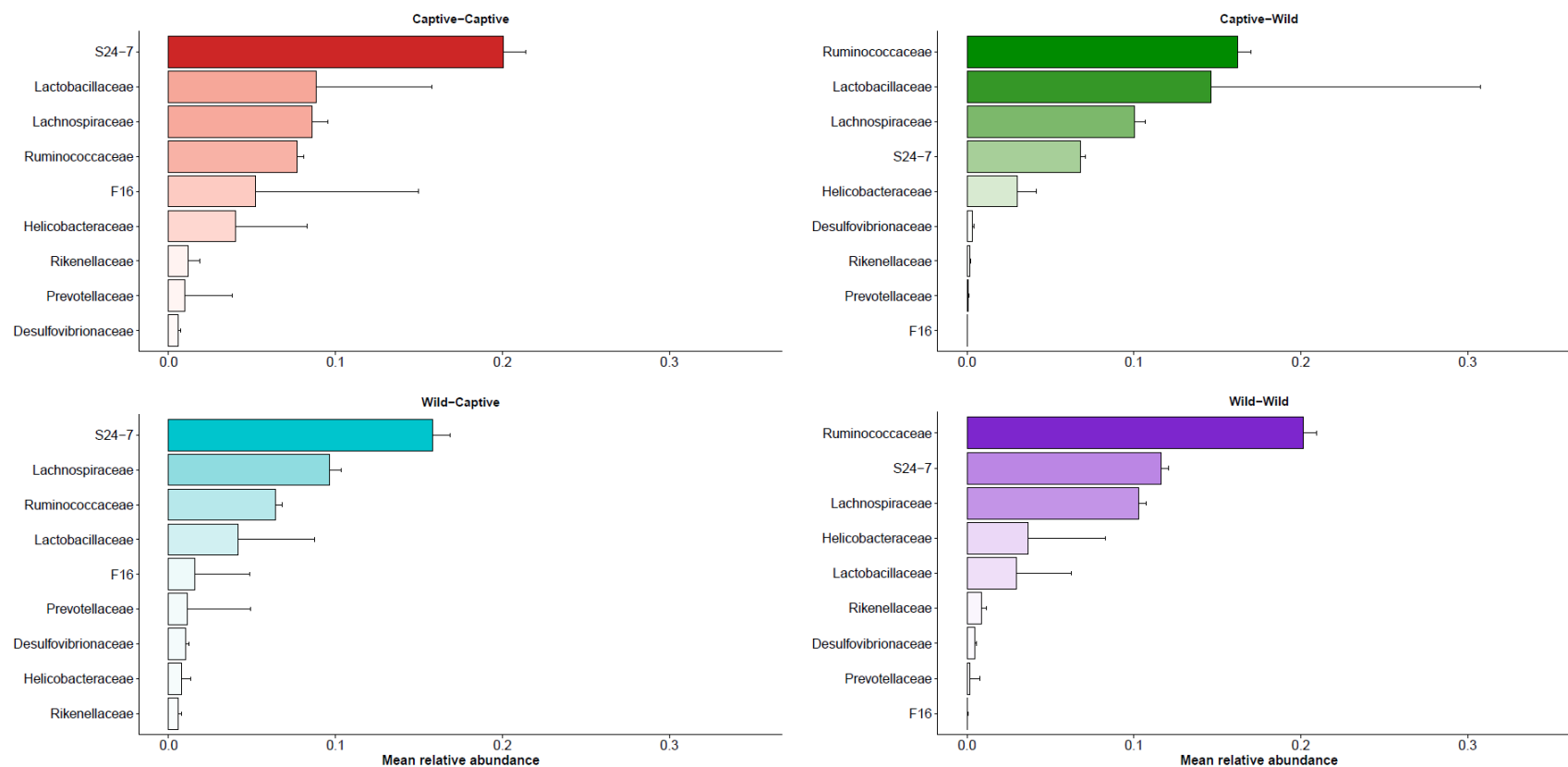


Figure 1-13: Mean relative abundance of different microbial families, which represented at least >1% or more of the total GMC within at least one individual deer mouse during the post-translocation sampling period. The microbial family *S24-7* was the most dominant in deer mice that were in captivity during the two-week translocation phase of the experiment (Captive-Captive and Wild-Captive groups). Alternatively, *Ruminococcaceae* was the most dominant microbial family in the GMC of deer mice who were in the wild for the translocation phase of the experiment (Wild-Wild and Captive-Wild groups).

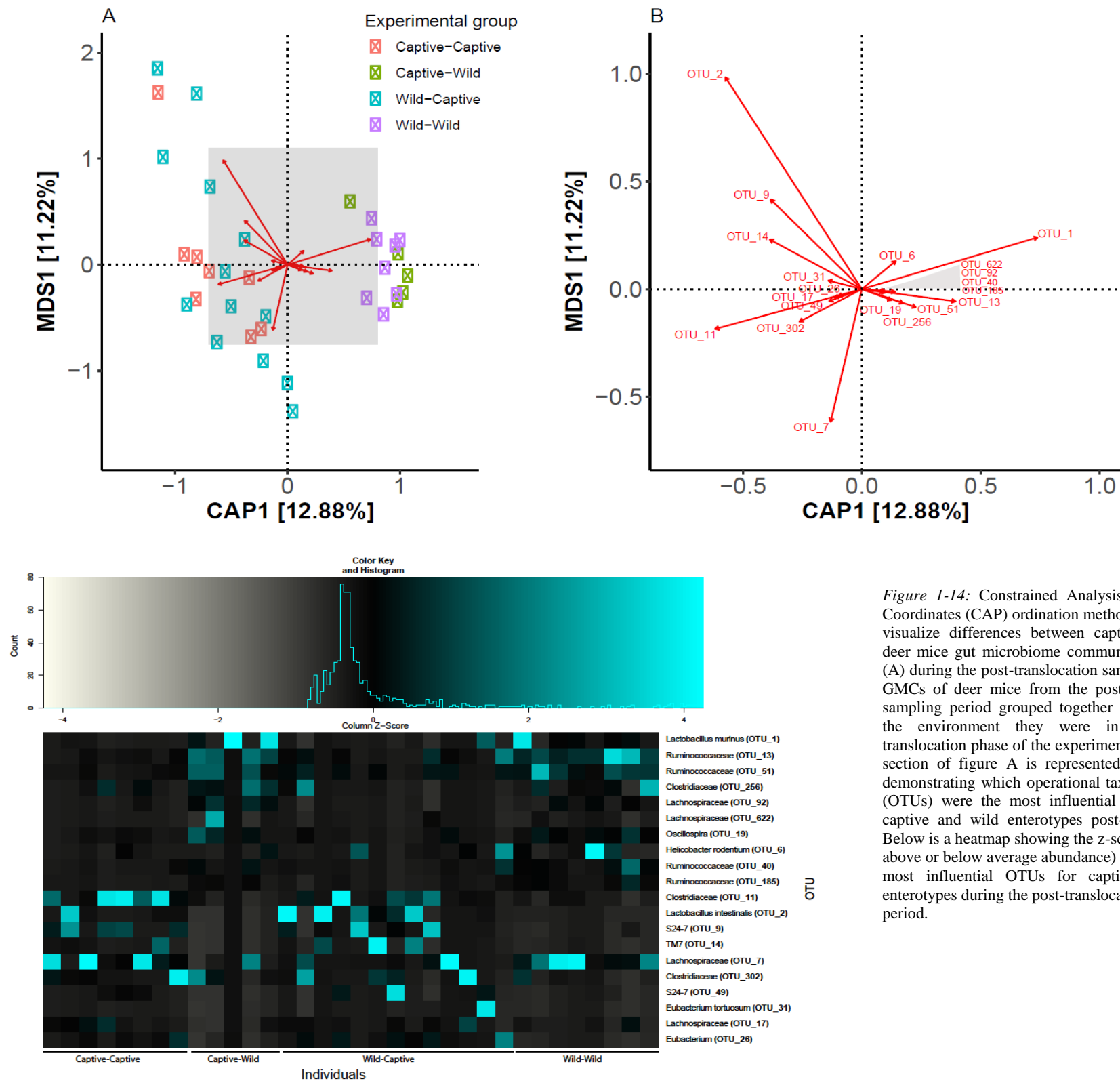


Figure 1-14: Constrained Analysis of Principle Coordinates (CAP) ordination method was used to visualize differences between captive and wild deer mice gut microbiome communities (GMCs) (A) during the post-translocation sampling period. GMCs of deer mice from the post-translocation sampling period grouped together depending on the environment they were in during the translocation phase of the experiment. The shaded section of figure A is represented in figure B, demonstrating which operational taxonomic units (OTUs) were the most influential in separating captive and wild enterotypes post-translocation. Below is a heatmap showing the z-scores (indicate above or below average abundance) for the top ten most influential OTUs for captive and wild enterotypes during the post-translocation sampling period.

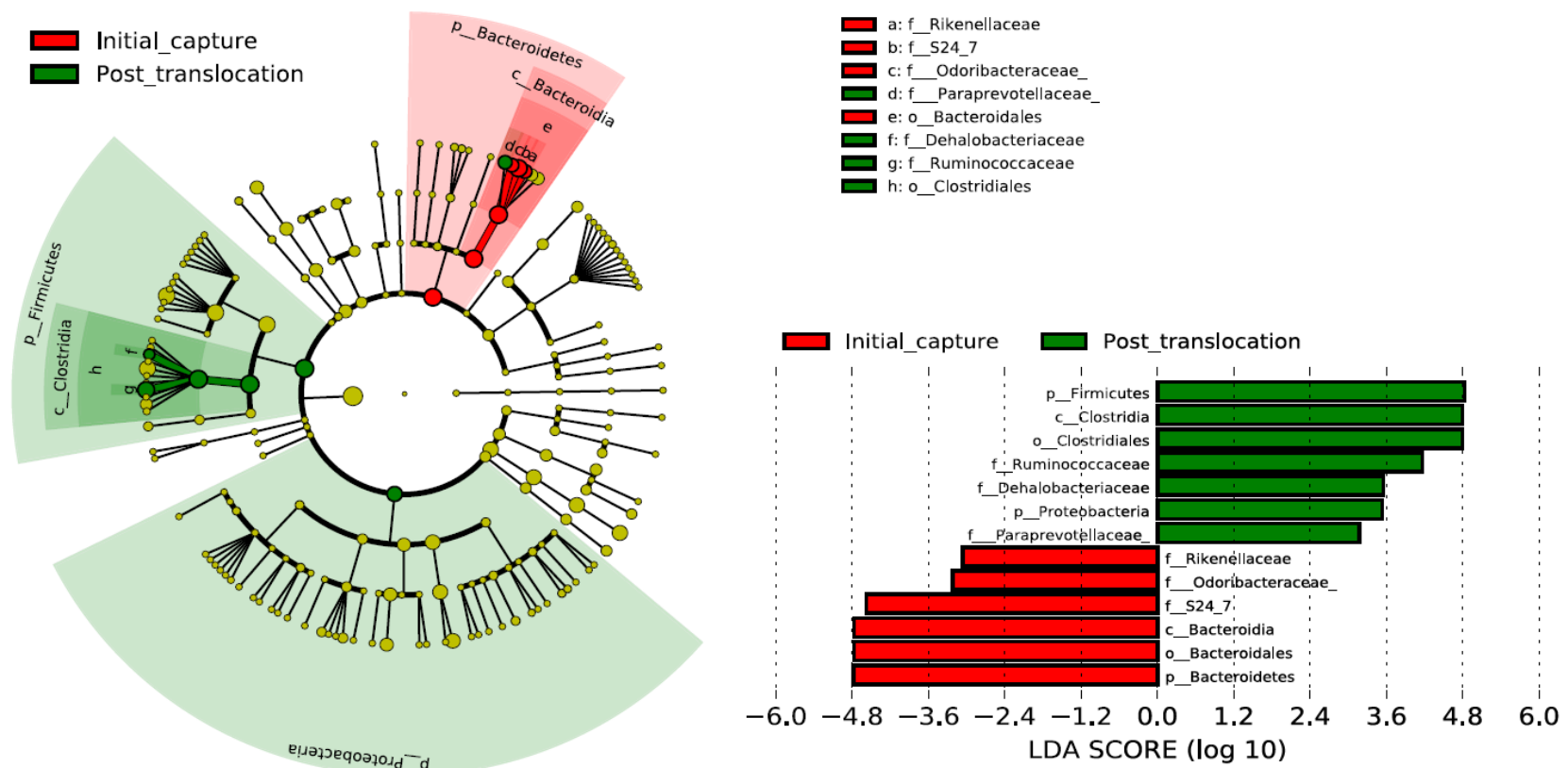


Figure 1-15: Linear discriminant analysis (LDA) effect size (LEfSe) determined that microbial families including *Rikenellaceae*, *Odoribacteraceae* and *S24-7* were more abundant during the initial sampling period, compared to the post-translocation sampling period, in deer mice that were translocated from captivity to the wild (C-W). During the post-translocation sampling period microbial families including *Dehalobacteriaceae*, *Paraprevotellaceae* and *Ruminococcaceae*, as well as the phylum *Proteobacteria* were significantly more abundant in C-W deer mice GMCs compared to C-W GMC samples acquired during the initial sampling period.

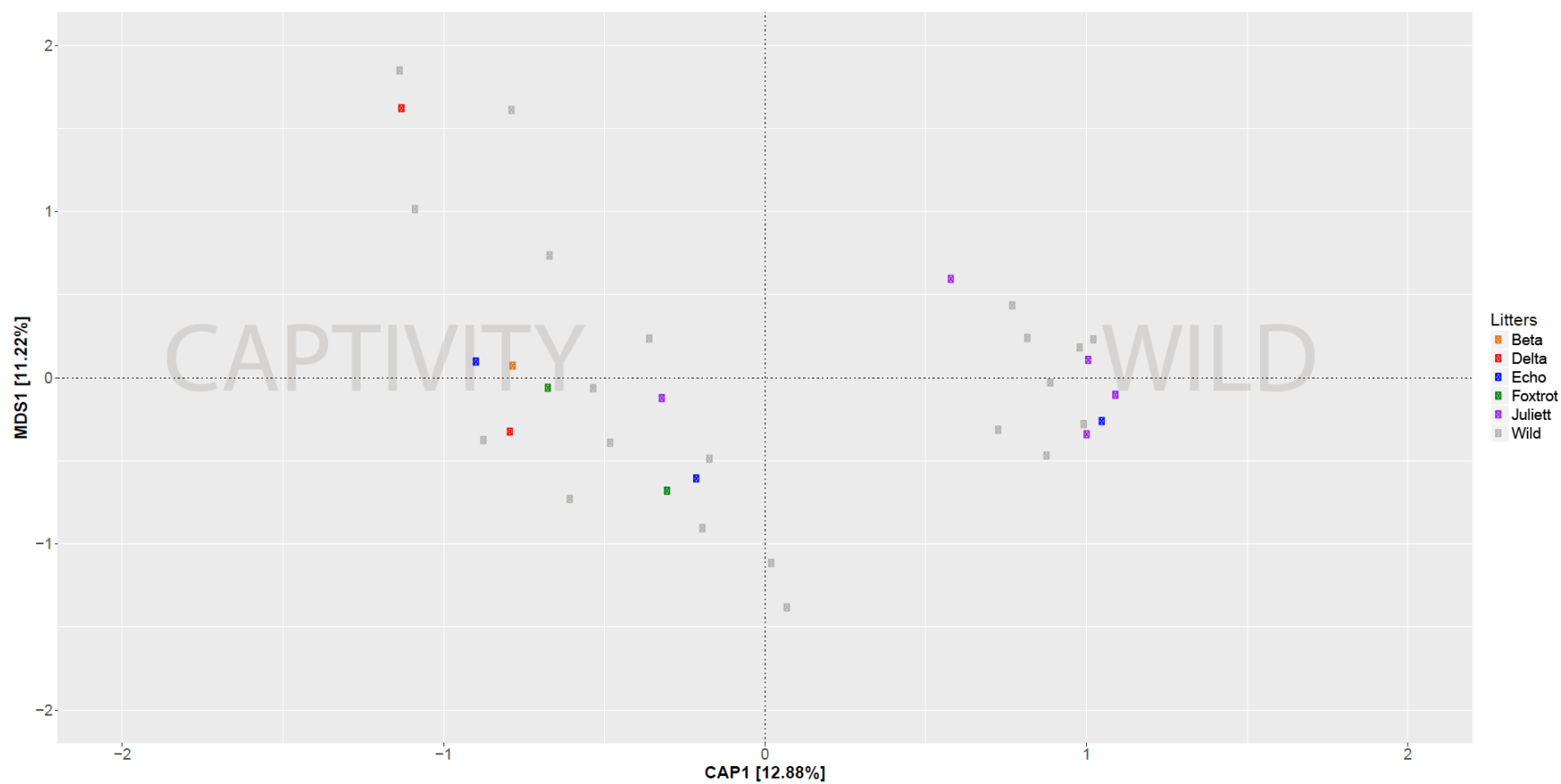


Figure 1-16: Constrained Analysis of Principle Coordinates (CAP) ordination method was used to visualize the difference between captive and wild born deer mice post-translocation. Each color corresponds to a different litter that was born in captivity, while individuals born in the wild are all colored grey since their dams are unknown. There does not appear to be any clear clustering between littermates in either captivity or the wild post-translocation.

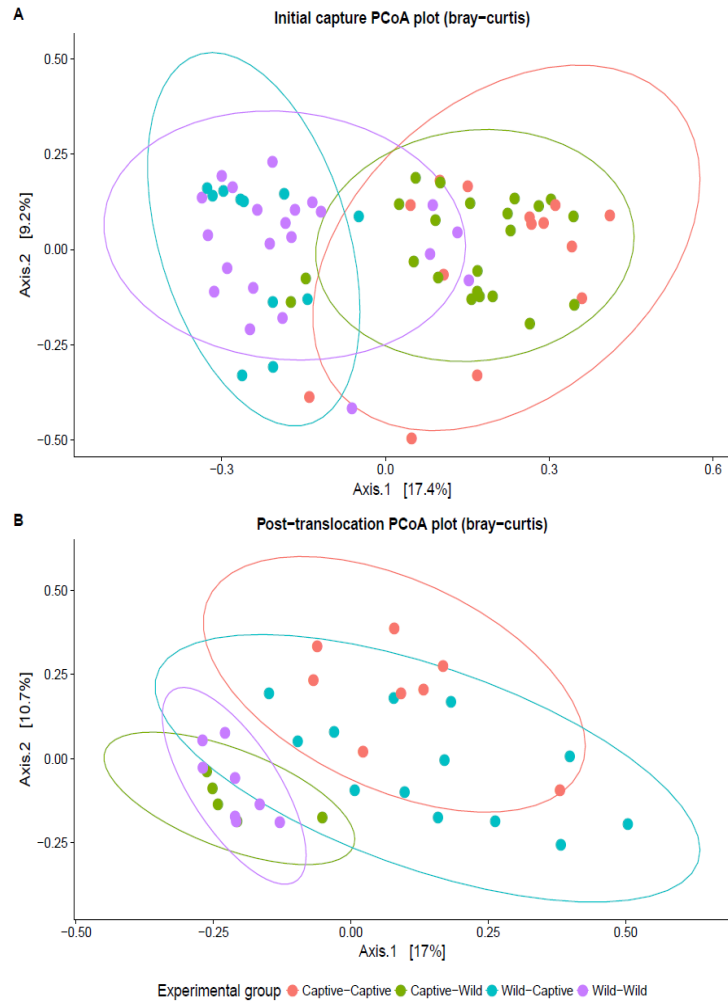
Supplementary Table 1-1: Mean relative abundance of microbes at different taxonomic classification levels. Non-parametric unpaired Mann-Whitney U tests were performed to test for significant differences between deer mice born in captivity versus individuals the wild. Significant differences were seen for a number of microbes at different taxonomic levels.

Taxonomic level	Mean Relative Abundance			<i>p-value</i>
	Captive	Wild	Δ between captive and wild	
Phylum				
Firmicutes	41.13	68.39	+27.26	<0.001
Bacteroidetes	50.22	20.72	-29.5	<0.001
Proteobacteria	5.50	8.52	+3.02	<0.001
TM7	1.98	0.21	-1.77	<0.001
Class				
Clostridia	32.04	58.05	+26.01	<0.001
Bacteroidia	50.03	20.02	-30.01	<0.001
Bacilli	8.94	9.84	+0.9	0.90
Epsilonproteobacteria	1.68	6.01	+4.33	<0.01
Gammaproteobacteria	3.45	1.12	-2.33	<0.05
TM7-3	1.98	0.09	-1.89	0.99
Deltaproteobacteria	0.26	1.01	+0.75	<0.001
Order				
Clostridiales	32.03	58.04	+26.01	<0.001
Bacteroidales	50.03	20.02	-30.01	<0.001
Lactobacillales	8.93	9.80	+0.87	0.97
CW040	1.92	0.09	-1.83	0.99
Campylobacteriales	1.68	6.01	+4.33	<0.01
Desulfovibrionales	0.26	1.00	+0.74	<0.001
Enterobacteriales	3.44	0.26	-3.18	<0.001
Family				
S24-7	33.60	13.61	-19.99	<0.001
Ruminococcaceae	5.44	16.26	+10.82	<0.001
Lactobacillaceae	8.93	9.75	+0.82	0.98
Lachnospiraceae	6.25	8.68	+2.43	<0.01
Helicobacteraceae	1.67	6.01	+4.34	<0.01
Enterobacteriaceae	3.44	0.26	-3.18	<0.05
F16	1.90	0.093	-1.807	0.99

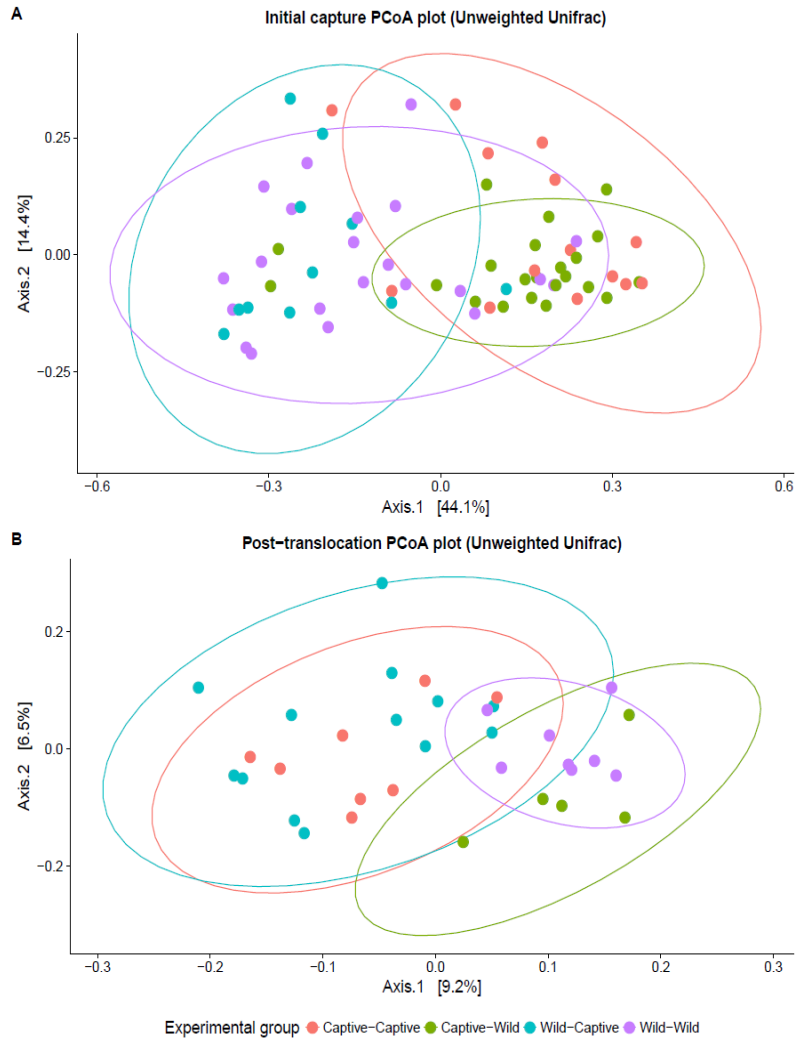
Supplementary Table 1-2: List containing the mean relative abundance of bacteria at the phylum, class, order and family level, during the post-translocation sampling stage for deer mice in all four experimental groups.

Taxonomic level	Mean Relative Abundance (% of gut microbiome community)				<i>p-value</i>	Post-hoc
	Captive-Captive	Captive-Wild	Wild-Captive	Wild-Wild		
Phylum						
Firmicutes	73.24	84.27	60.02	74.97	p<0.05*	Wild-Lab<Lab-Wild
Bacteroidetes	21.41	10.72	26.00	18.49	p=0.10	
Proteobacteria	2.65	3.67	6.724	4.49	p=0.71	
TM7	1.56	0.037	5.19	0.037	p=0.15	
Class						
Clostridia	68.86	69.36	48.64	71.71	p=0.22	
Bacteroidia	21.33	10.69	25.91	17.95	p=0.10	
Bacilli	4.21	1.46	8.91	3.00	p=0.87	
Epsilonproteobacteria	0.78	3.04	4.00	3.66	p=0.092	
Gammaproteobacteria	0.0053	0.0042	1.56	0.038	p=0.61	
TM7-3	1.56	0.0015	5.19	0.037	p=0.15	
Deltaproteobacteria	1.24	0.45	0.65	0.71	p=0.76	
Order						
Clostridiales	68.86	69.36	48.63	71.69	p=0.22	
Bacteroidales	21.33	10.69	25.91	17.95	p=0.10	
Lactobacillales	4.18	14.60	8.89	2.99	p=0.85	
CW040	1.56	0.015	5.19	0.037	p=0.74	
Campylobacterales	0.78	1.11	4.00	3.66	p=0.15	
Desulfovibrionales	1.24	0.45	0.71	0.65	p=0.092	
Enterobacteriales	0.0034	0	1.55	0.031	p=0.33	
Family						
S24-7	15.83	6.82	20.07	11.61	p=0.081	Lab-Lab<Wild-Wild Wild-Lab<Wild-Wild
Ruminococcaceae	6.40	16.23	7.69	20.13	p<0.001*	
Lactobacillaceae	4.18	14.6	8.86	2.99	p=0.82	
Lachnospiraceae	9.64	10.04	8.61	10.28	p=0.65	
Helicobacteraceae	0.78	3.04	4.00	3.66	p=0.092	
Desulfovibrionaceae	1.03	0.33	0.58	0.473	p=0.73	
F16	1.56	0.015	5.19	0.037	p=0.15	
Prevotellaceae	1.11	0.052	0.98	0.154	p=0.41	
Rikenellaceae	0.57	0.172	1.17	0.89	p<0.05*	
Enterobacteriaceae	0.0034	0	1.55†	0.00088	p=0.33	Wild-Lab<Lab-Wild Wild-Wild<Lab-Wild

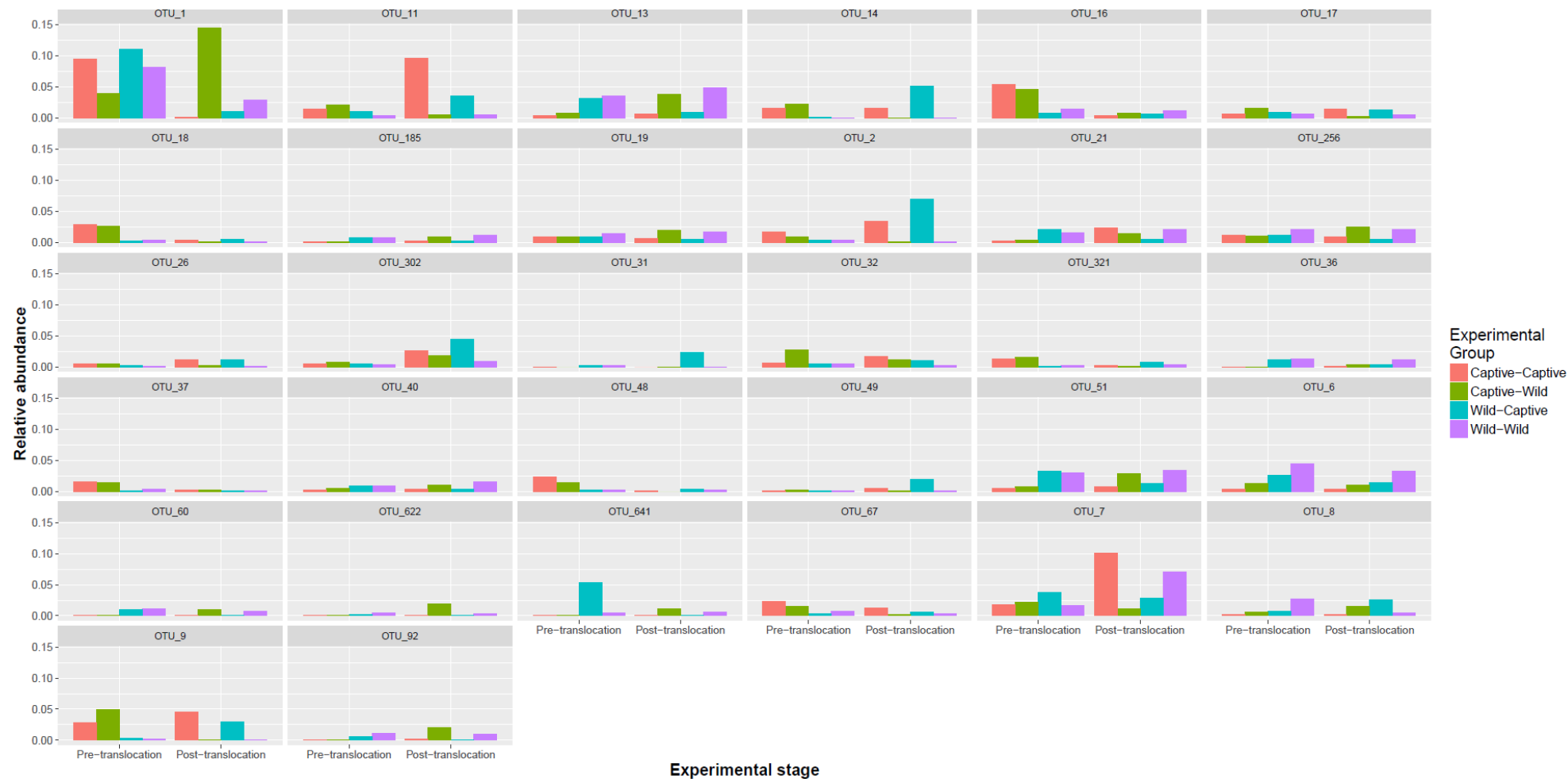
Supplementary figures:



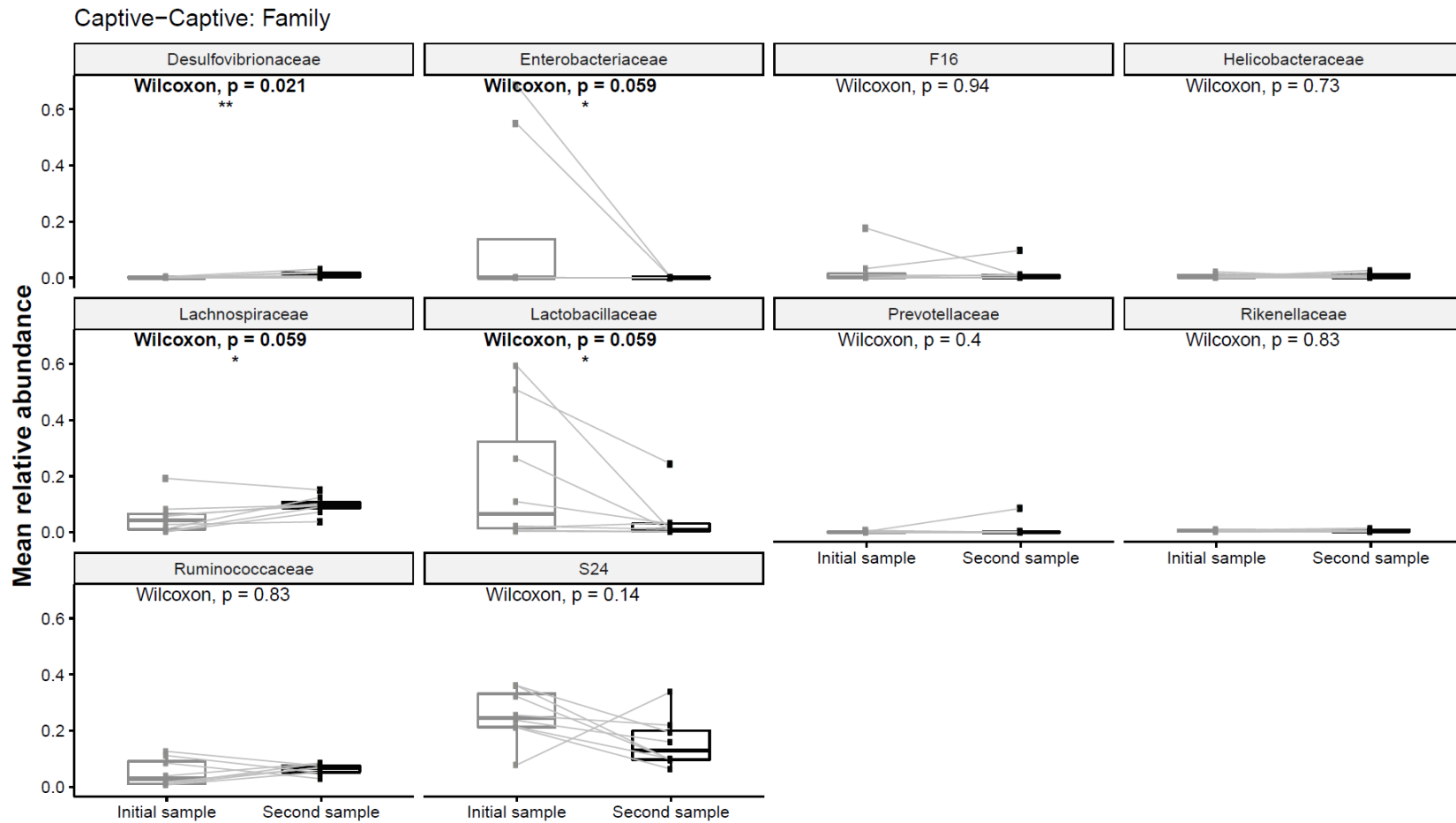
Supplementary Figure 1-1: Principle Coordinate Analysis ordinations using Bray-Curtis dissimilarity measurements demonstrate that A) deer mice born in the captive or wild environments cluster together with deer mice born in the same environment (Adonis: $F_{1,64} = 3.80$, $R^2 = 0.15$, $p < 0.001$) and B) post-translocation deer mice cluster closer with individuals sharing the same environment rather than where they were born (Adonis: $F_{1,33} = 4.81$, $R^2 = 0.13$, $p < 0.001$).



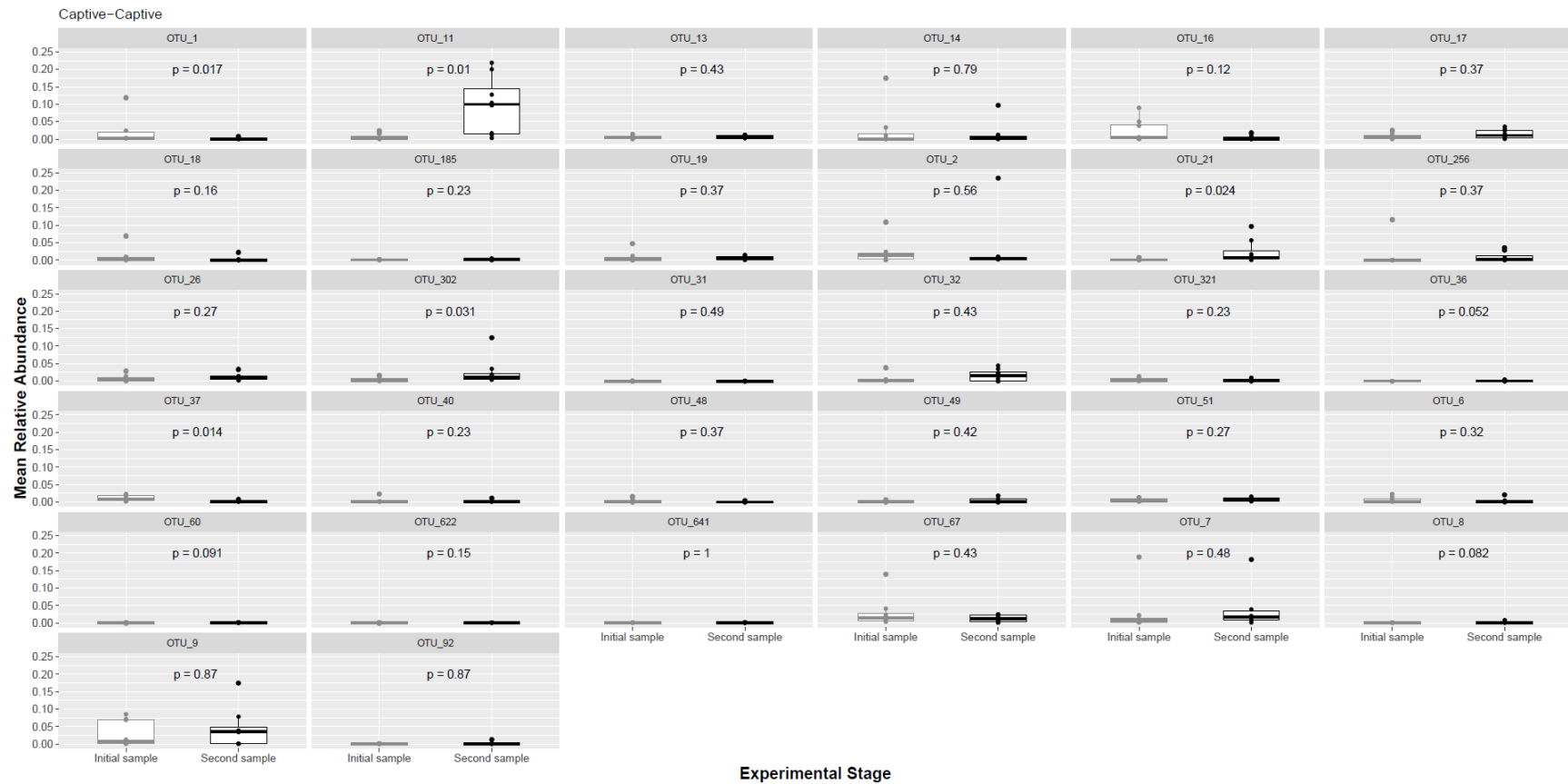
Supplementary Figure 1-2: Principle Coordinate Analysis ordinations using unweighted UniFrac measurements show that the GMC of deer mice individuals born in the same environment (A) cluster together (adonis: $F_{1,64} = 3.20$, $R^2 = 0.13$, $p < 0.001$), however, during the post-translocation stage (B) the external environment surrounding deer mice determined which individuals clustered closer together based on GMC composition (Adonis: $F_{1,33} = 2.68$, $R^2 = 0.07$, $p < 0.001$).



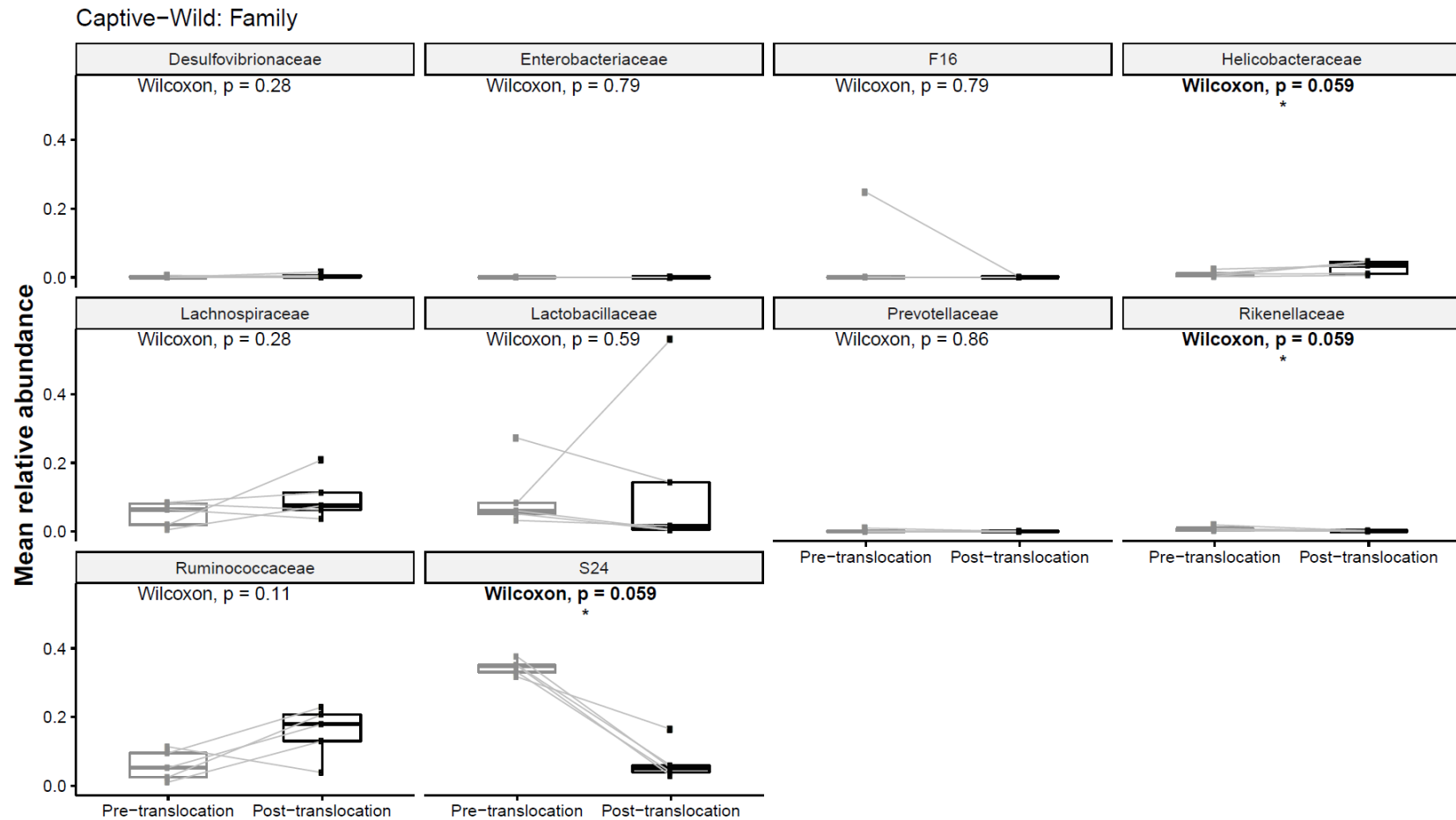
Supplementary Figure 1-3: The abundance of OTUs which were determined to be enterotype-defining during either the pre-translocation or post-translocation sampling periods. All OTUs had a mean relative abundance $\geq 1\%$ in at least one of the four groups during either the pre- or post-translocation sampling period. A number of OTUs are shown to demonstrate a strong relationships with the natural environment, represented by high mean relative abundances within Wild-Captive and Wild-Wild groups during the pre-translocation sampling period and high mean relative abundances in Captive-Wild and Wild-Wild groups post-translocation.



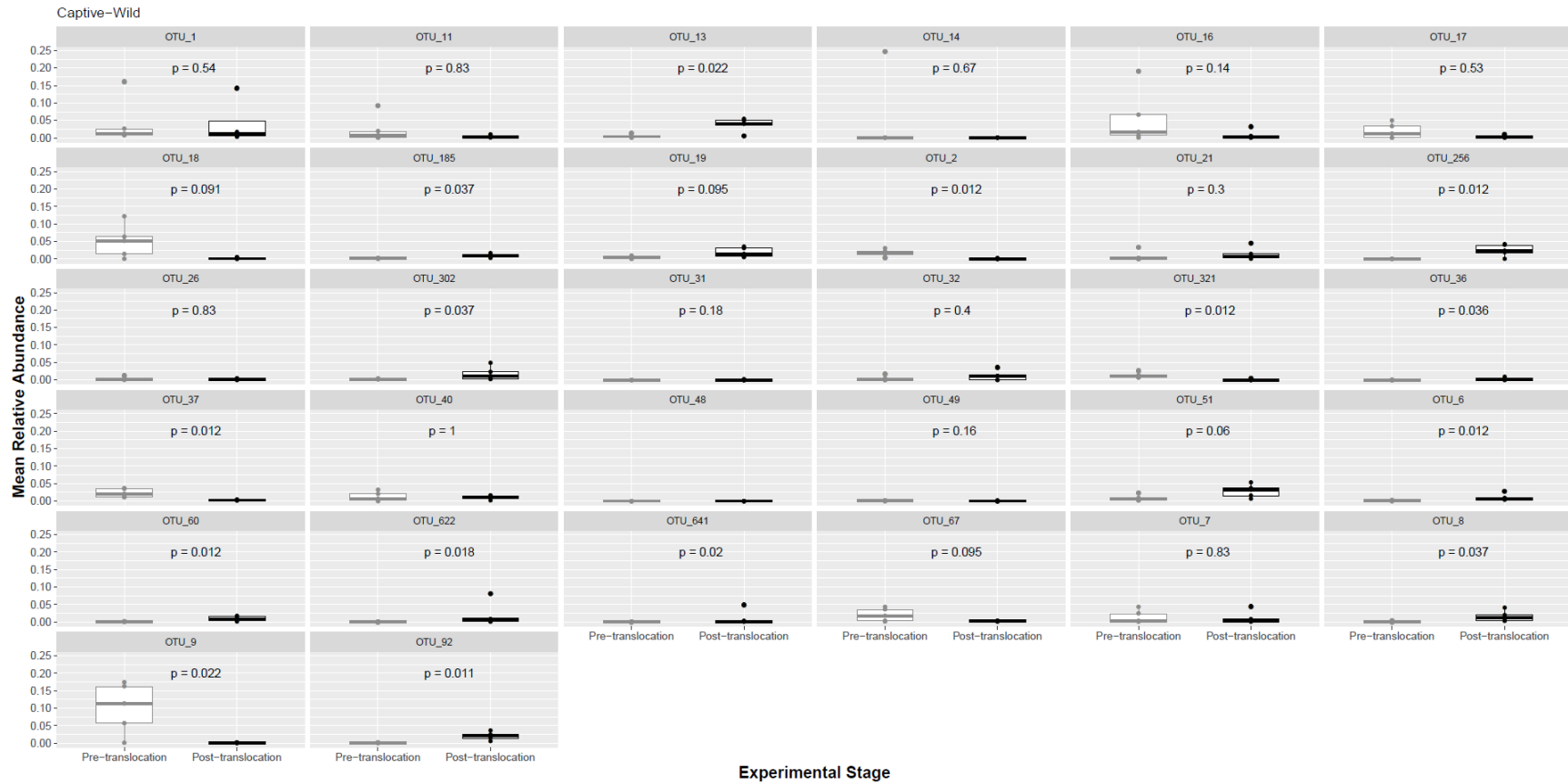
Supplementary Figure 1-4: Pairwise analysis of individuals from the Captive-Captive group, examining the change in relative abundance of families which make up at least 1% of the gut microbiome community (in at least one individual). Significant difference was seen in the family *Desulfovibrionaceae* ($p = 0.021$) and marginal differences in *Enterobacteriaceae* ($p = 0.059$)†, *Lachnospiraceae* ($p = 0.059$) and *Lactobacillaceae* ($p = 0.059$). †This marginal decrease seems to be largely driven by only two individuals in the group. ** indicates $p < 0.05$ and * indicates $p < 0.1$.



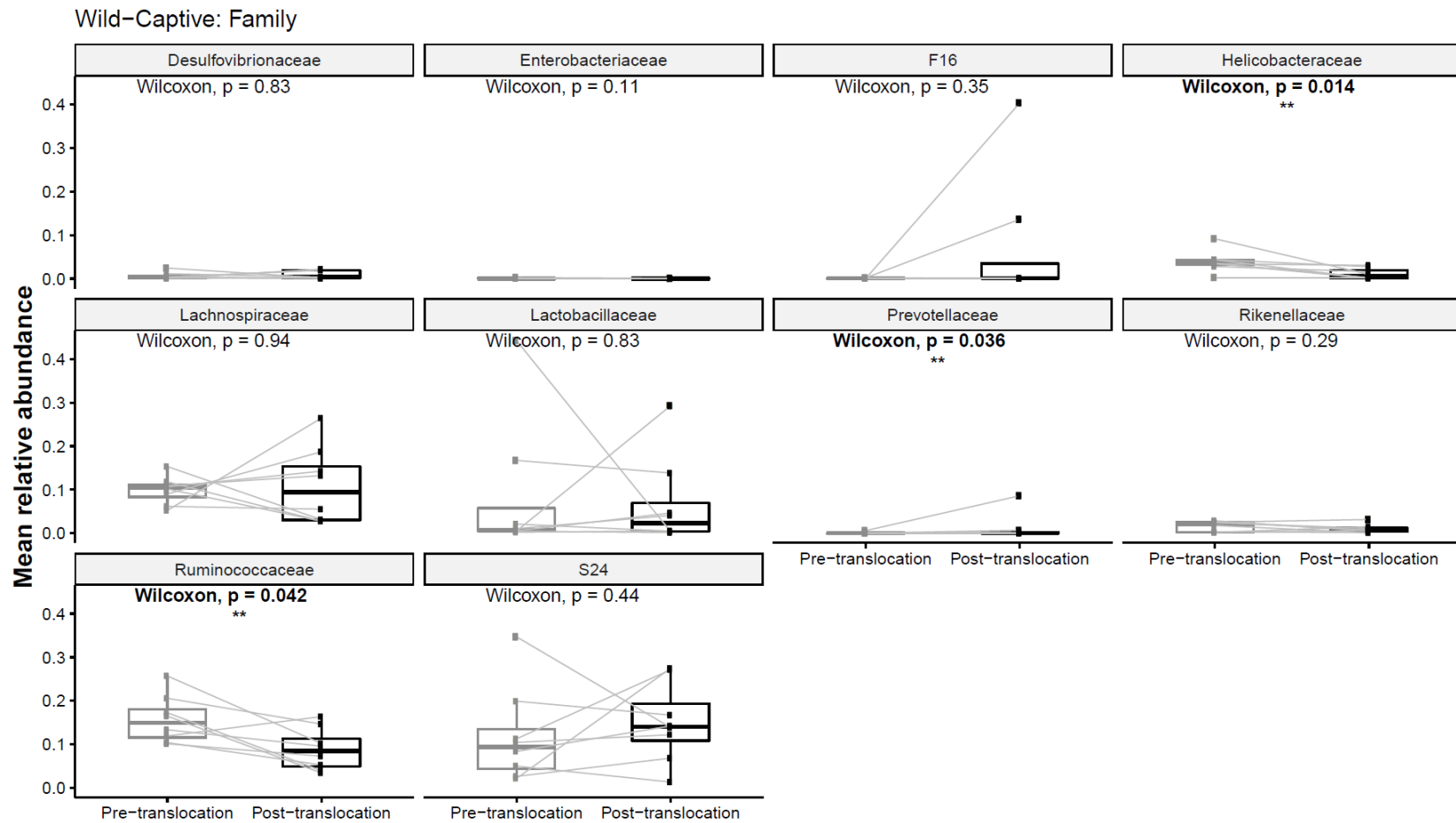
Supplementary Figure 1-5: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be important during the initial or post-translocation sampling periods. Deer mice in the Captive-Captive group demonstrated significant decreases in OTU 1 (*Lactobacillus murinus*, $p = 0.017$), and OTU 37 (*S24-7*, $p = 0.014$). Significant increases were found for OTU 11 (*Clostridiaceae*, $p = 0.01$), OTU 21 (*Lachnospiraceae*, $p = 0.024$), and OTU 302 (*Clostridiaceae*, $p = 0.031$).



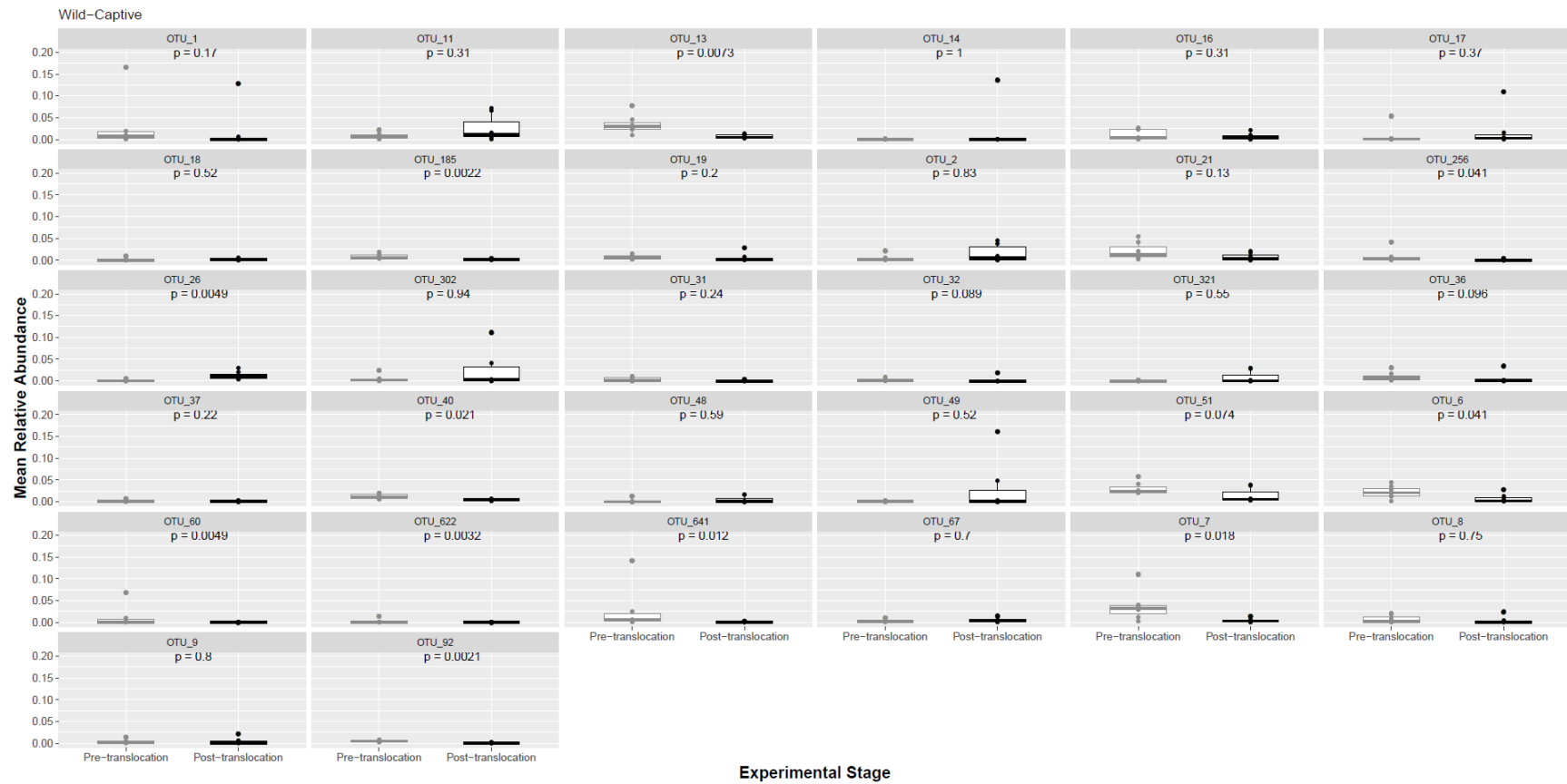
Supplementary Figure 1-6: Wilcoxon paired analysis was used to examine the change in relative abundance of families which made up at least 1% of the gut microbiome community (in at least one deer mouse) of deer mice from the Captive-Wild group. Marginally significant differences were seen in the microbial families *Helicobacteraceae* ($p = 0.059$), *Rikenellaceae* ($p = 0.059$), and *S24-7* ($p = 0.059$). ** indicates $p < 0.05$ and * indicates $p < 0.1$.



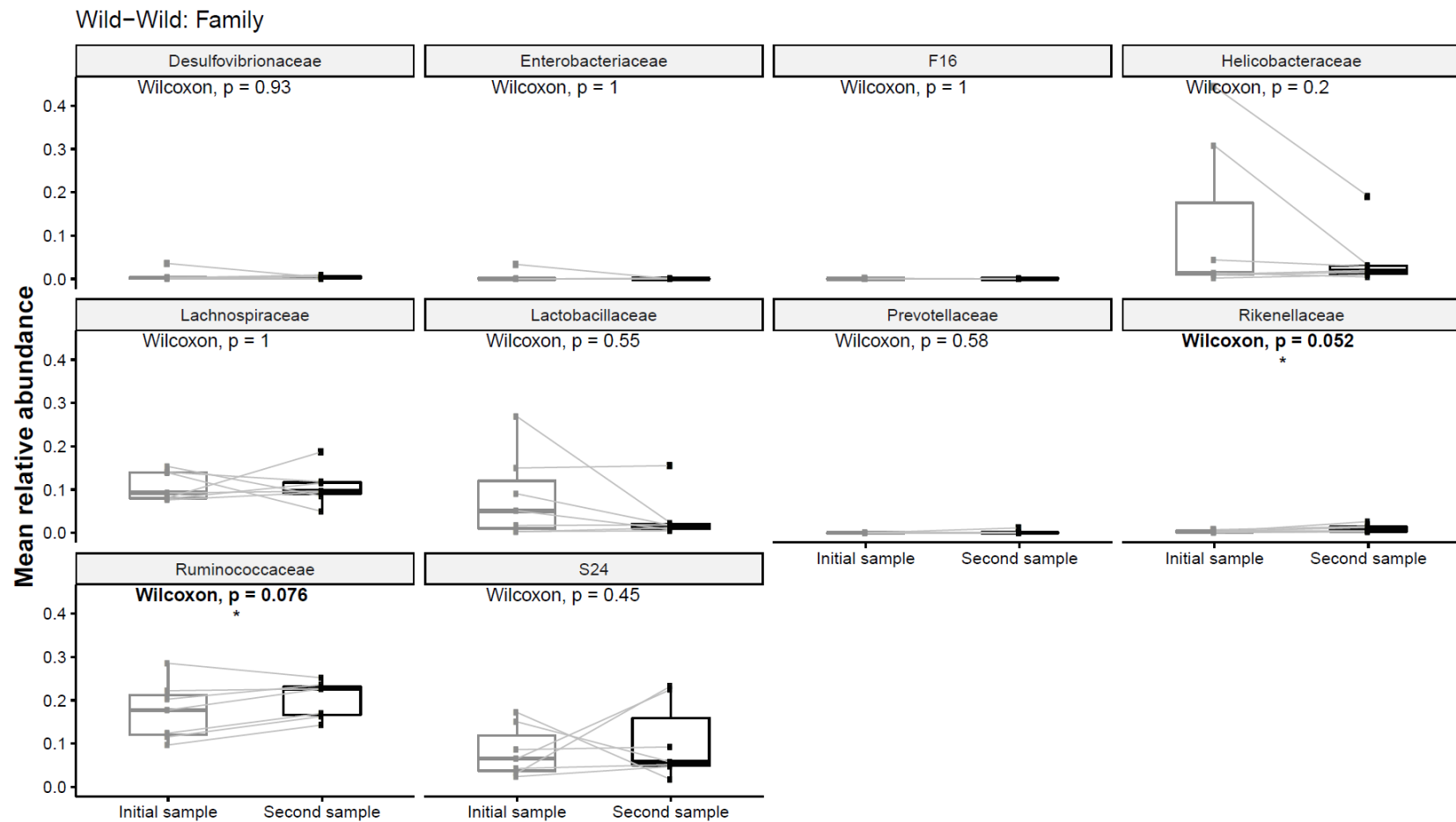
Supplementary Figure 1-7: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be characteristic of captive and wild enterotypes during the initial and second (post-translocation) sampling period was conducted to assess significant changes within the gut microbiome communities of deer mice in the Captive-Wild group. Significant increases ($p < 0.05$) were found for OTU 13 (*Ruminococcaceae*), OTU 185 (*Ruminococcaceae*), OTU 256 (*Clostridiaceae*), OTU 302 (*Clostridiaceae*), OTU 36 (*Clostridiaceae*), OTU 6 (*Helicobacter rodentium*), OTU 60 (*Lachnospiraceae*), OTU 622 (*Lachnospiraceae*), OTU 641 (*Lachnospiraceae*), OTU 8 (*Helicobacter aurati*) and OTU 92 (*Lachnospiraceae*), with OTU 51 (*Ruminococcaceae*, $p = 0.06$) also showing a near significant increase. Significant decreases ($p < 0.05$) were seen among OTU 2 (*Lactobacillus intestinales*), OTU 321 (*S24-7*), OTU 37 (*S24-7*), and OTU 9 (*S24-7*).



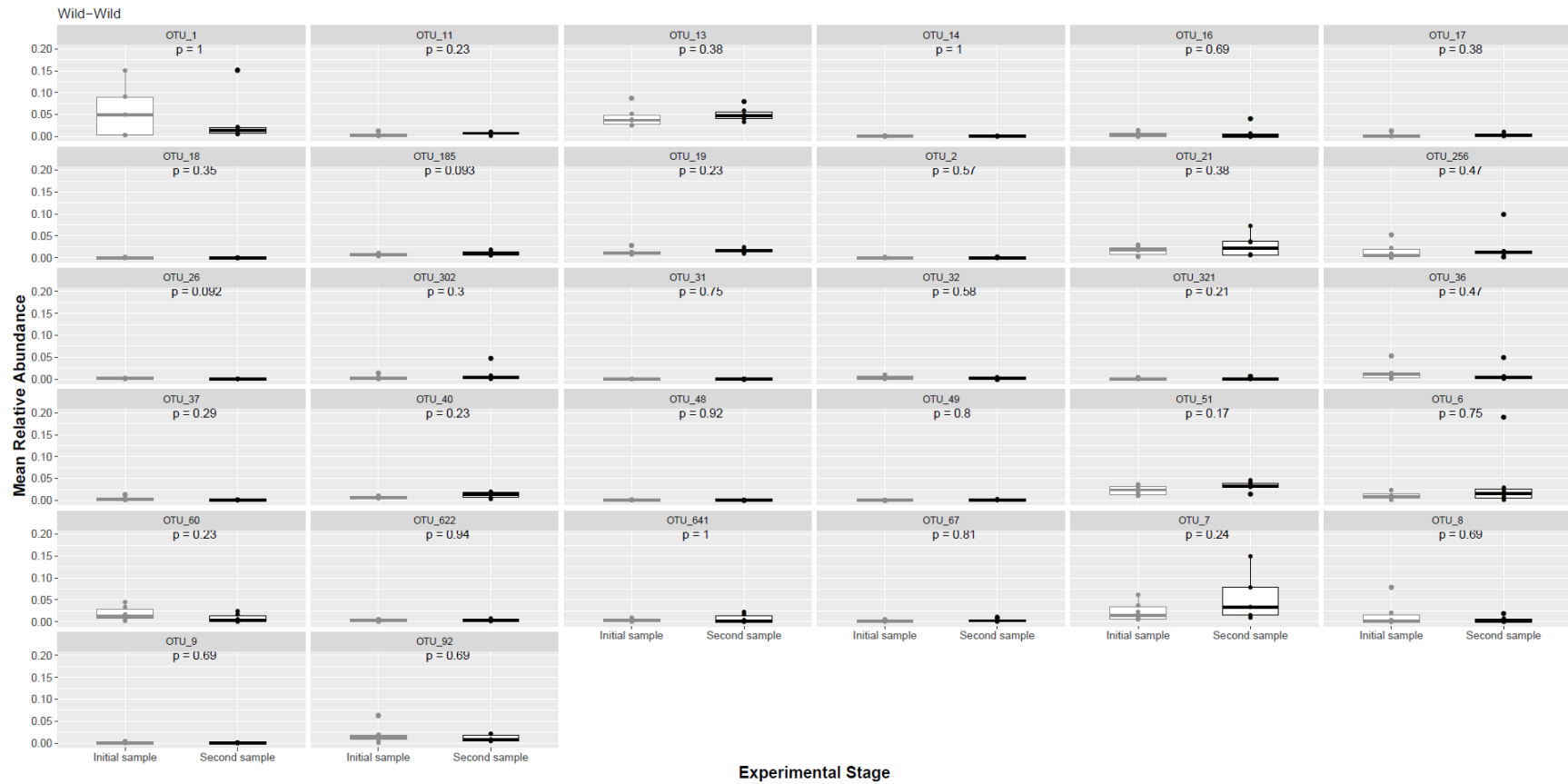
Supplementary Figure 1-8: Wilcoxon paired analysis was used to examine the change in relative abundance of Families which made up at least 1% of the gut microbiome community (in at least one deer mouse) of deer mice from the Wild-Captive group. Significant difference in were seen in Families *Helicobacteraceae* ($p = 0.014$), *Prevotellaceae* ($p = 0.036$), and *Ruminococcaceae* ($p = 0.042$). ** indicates $p < 0.05$ and * indicates $p < 0.1$.



Supplementary Figure 1-9: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be characteristic of captive and wild enterotypes during the initial and post-translocation sampling period was conducted to assess significant changes within the gut microbiome communities of deer mice in the Wild-Captive group. Significant increases ($p < 0.01$) was only found for OTU 26 (*Eubacterium*). Significant decreases ($p < 0.01$) were seen among OTU 13 (*Ruminococcaceae*), OTU 185 (*Ruminococcaceae*), OTU 60 (*Lachnospiraceae*), OTU 622 (*Lachnospiraceae*) and OTU 92 (*Lachnospiraceae*) as well as ($p < 0.05$ for the following) OTU 256 (*Clostridiaceae*), OTU 40 (*Ruminococcaceae*) and OTU 641 (*Lachnospiraceae*).



Supplementary Figure 1-10: Wilcoxon paired analysis was used to examine the change in relative abundance of families which made up at least 1% of the gut microbiome community (in at least one deer mouse) of deer mice from the Wild-Wild group. Only a marginal difference was seen in *Rikenellaceae*. ** indicates $p < 0.05$ and * indicates $p < 0.1$.



Supplementary Figure 1-7: Wilcoxon paired analysis of Operational Taxonomic Units (OTUs) determined to be characteristic of captive and wild enterotypes during the initial and post-translocation sampling period was conducted to assess significant changes within the gut microbiome communities of deer mice in the Wild-Wild group. No significant changes were seen among any of the top influential OTUs in deer mice in the Wild-Wild group.

Chapter 2: Relationships between gut microbiome α -diversity and host immunocompetence

2.1 Abstract

Microbial communities inhabiting the gastrointestinal tracts of vertebrates have co-existed with their hosts over millions of years, forming commensal, pathogenic, and symbiotic relationships. Gut microbiota provide their host with a number of beneficial advantages and collaborate in early life physiological development. One of the most important microbe-host collaborations is the development of the host's immune system. Previous studies have compared gut microbiome diversity between captive and wild individuals in a number of taxa, however, few studies have attempted to correlate microbial differences with potential fitness consequences. This is one of the first studies to assess environment-microbe-host interactions using an eco-immunological approach to assess fitness consequences associated with gut microbiome alterations. Over the course of this study captive and wild deer mice (*Peromyscus maniculatus*) were sampled twice, two-weeks apart. During each sampling period fecal and blood samples (via tail snip) were collected. Fecal samples were used to examine gut microbiome diversity using 16S rRNA gene next-generation Illumina HiSeq sequencing. Blood samples were used for immunological measurements including, proxies for basal immune investment (hematocrit ratios and white blood cell counts and differentials) as well as functional immune responses (i.e. bacteria killing ability [BKA]). Although individuals in the wild had a marginally increased BKA, no direct correlation was found with gut microbiome diversity. A marginal correlation was found between the change in α -diversity (Shannon's index) and change in BKA between sampling periods. Marginal associations were found between α -diversity and measurements representing proxies for basal immune investment, while hematocrit ratios showed no relationship with microbial diversity. Our findings demonstrate that an individual's gut microbiome is heavily influenced by their surrounding environment, however does not contain strong correlations with immune measurements.

2.2 Introduction

Vertebrate species are hosts to trillions of microbes, which inhabit various parts of their host's bodies, making up unique and highly adapted communities (Sommer and Bäckhed 2013). These communities are referred to as microbiomes and represent a host's microbial genome. One of the most influential microbiomes among vertebrate hosts is the gastrointestinal (gut) microbiome, which is thought to have originated through mutualistic relationships, with microbes providing their host's with the ability to digest previously inaccessible food sources (e.g. plant material), and hosts providing microbes with a habitat high in nutrient content (Hooper 2004, O'Hara and Shanahan 2006, Ley et al. 2008a, 2008b, Round and Mazmanian 2009). Additionally, gut microbes contribute to their host's physiological development, including immune system development and maturation (Kelly et al. 2007, Round and Mazmanian 2009, Lee and Mazmanian 2010, Mulder et al. 2011, Purchiaroni et al. 2013, Thaïss et al. 2014).

Host immune systems evolved to be able to identify and differentiate between harmful and beneficial microbes to maximize host fitness (Kelly et al. 2007, Neu 2007, Mulder et al. 2011). The gastrointestinal tract is an important interaction site where cross-talk has been established between gut microbes and the host's immune system (Neu 2007, Round and Mazmanian 2009, Cerf-Bensussan and Gaboriau-Routhiau 2010, Cahenzli et al. 2013, Shapiro et al. 2014), resulting in interdependent, feedback mechanisms and development (Round and Mazmanian 2009, Thaïss et al. 2014). Complex gut microbe-immune system interactions are developed during early life periods (Ley et al. 2008b), making early life phases critical periods for microbe establishment within their hosts, a process which can be heavily influenced by the exposure to the extrinsic environment (Neu 2007, Mulder et al. 2009, 2011).

Evidence of environment-microbe-immune interactions was first proposed under the ‘hygiene hypothesis’, which states that early life exposure to microbes is essential for the proper development and maturity of an individual’s immune system (Strachan 1989). Empirical evidence of the ‘hygiene hypothesis’ has been provided by studies comparing vaginal and C-section born infants, showing the latter often develop inadequate symbiotic relationships with colonizing microbes, leading to increased health issues including, pathogen overgrowth, increased disease expression and susceptibility to allergy and autoimmune disorders (e.g. allergies, asthma, irritable bowel disorder) later in life (Weng and Walker 2013, Dominguez-Bello et al. 2016). These study provide evidence that increased microbial exposure and diversity can have strong influences on host immune system development.

Numerous studies comparing captive and wild individuals have determined that captive individuals typically possess different and less diverse gut microbiome communities (GMCs) compared to their wild counterparts (Villers et al. 2008, Xenoulis et al. 2010, Nakamura et al. 2011, Kohl et al. 2014, Cheng et al. 2015, see *Chapter 1*). Phylum level differences as well as intra-genus specialization have been demonstrated within captive, wild (see *Chapter 1*) and lab strains of murine (Schloss et al. 2012). However, the effect of large or subtle changes within the gut microbiome on host fitness is poorly understood. Gut microbiome-host research has been dominated by lab-based or germ-free studies, with little research attempting to understand these relationships in more natural settings, where the influence of gut microbes on host health may be diluted by other factors such as: food availability, parasite load, and stress (Grenham et al. 2011, Kohl and Dearing 2014, Kreisinger et al. 2014, Hird 2017).

Extrapolating findings from studies conducted in controlled laboratory conditions to wild populations should be done cautiously, due to the influence the exogenous environment may have

on GMCs (see *Chapter 1*). However, laboratory based studies have provided evidence that GMCs may influence their host's health and immune system development (Stappenbeck et al. 2002, Mulder et al. 2009, 2011, Chung et al. 2012). Previous studies on piglets (*Sus scrofa*) demonstrated that reduced early life microbial exposure influences gut microbiome community establishment as well as immune system development (Mulder et al. 2009, 2011). Germ-free animals have been repeatedly shown to have reduced immune system function and development, resulting in increased susceptibility to infection, as well as inflammatory and autoimmune diseases compared to conventionally colonized individuals (Sprinz et al. 1961, Maier and Hentges 1972, Kelly et al. 2007, Round and Mazmanian 2009, Thaiss et al. 2014). The addition of bacteria to the gut of germ-free animals results in increased immune development and activity (Stappenbeck et al. 2002, Chung et al. 2012), reinforcing the interdependency between gut microbes and immune development. Studies using germ-free animals provide valuable information pertaining to the relationship between immunity and gut microbiome diversity, however, the applicability of these studies to natural populations are limited (Grenham et al. 2011).

Experiments involving natural populations are inherently more complex than laboratory based studies set under highly controlled conditions, however future research focusing on wild populations should attempt to gain a better understanding of microbe-host relationships and their potential fitness consequences. One possible solution is to compare microbiome data with fitness proxies using an eco-immunological approach. Eco-immunology is an important tool for environmentally-oriented biologists in helping understand an organism's immune response on an evolutionary, ecologically, and life-history scale, by focusing on assessment of an individual's ability to mount an effective immune response to a harmful substance, often referred to as an individual's immunocompetence (Demas et al. 2011). One approach to assessing

immunocompetence is through testing functional immune responses using a bacteria killing assay. One of the main advantages of using bacteria killing assays is the ability to provide a functionally relevant assessment of an individual's immunocompetence rather than an isolated component of the immune system (Demas et al. 2011).

Despite the recent interest in conducting microbiome studies using wild populations, little attention has been paid to examining correlations between microbial communities and fitness in non-laboratory populations. This study will provide one of the first examples where differences in GMCs are attempted to be correlated with immunocompetence, which could reveal potential fitness related consequences for host species. Results will provide evidence on whether or not animals in captivity are less immunocompetent than animals in the wild, due to having lower GMC α -diversity levels. This study analyzed correlations between the α -diversity of gut microbiomes of captive and wild born deer mice (*Peromyscus maniculatus*), with haematological (i.e. hematocrit as well as total and differential white blood cell counts) and functional (i.e. bacteria killing assay) immune responses to investigate potential associations between gut microbiome diversity and host health. Gut microbiome α -diversity was hypothesized to be correlated with an individual's immunocompetence. Mice born in the wild and possess higher levels of α -diversity were predicted to possess optimal (i.e. closest to population mean [Bowers et al. 2014]) hematocrit, increased white blood cell and neutrophil:lymphocyte counts, as well as greater bacteria killing ability (BKA) compared to mice born in captivity whose gut microbiome communities are less diverse.

2.3 Methods

All methods in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Laurentian University, protocol number 2016-03-01.

2.3.1 Study site and sample collection

Deer mice were chosen as the most appropriate species for this study due to their high abundances, and ability to be easily handled and translocated. Deer mice were sampled from populations in Algonquin Provincial Park, Ontario, Canada (45°35'N, 78°31'W) using Longworth live traps (Rogers Manufacturing Co., Canada), between May and June.

The experimental design for this chapter was identical to that outlined in *Chapter 1* (section 1.3.2), except for the taking of blood samples, which is outlined in this chapter (see section 2.3.4). A summary of the experimental design is outlined below.

To obtain juvenile captive deer mice, pregnant deer mice were targeted and captured in May and early June. Female deer mice were determined to be pregnant based on weight, shape, and palpations. Upon capture pregnant females were transferred to a laboratory at the Algonquin Provincial Park Wildlife Research Station. Housing conditions for pregnant females are the same as those outlined in *Chapter 1*. All females and juveniles were provided with alphanumeric ear tags (National Brand and Tag co., Newport, KY, USA) so that deer mice could be identified.

Juvenile deer mice born in captivity were first sampled at three weeks of age after weaning. Fecal samples were collected directly from an individual's anus and blood samples (<100 µL) were collected via tail snips (for details see section 2.3.4). Prior to conducting tail snips, the tip of the tail was dipped in chilled isopropanol to desensitize the area. After collecting initial samples from deer mice born in captivity a subsample of individuals were translocated to the wild, while the remaining deer mice remained in captivity. Two weeks after the initial samples were acquired, individuals were sampled again to obtain an additional sample. By obtaining two different samples over a two-week period, it was possible to assess how changes in GMC α -diversity influences changes in host immunocompetence. Individuals translocated from captivity to the wild were transferred in wooden nest boxes (12.5 cm x 12.5 cm x 15 cm) filled with bedding material and

sunflower seeds. Nest boxes were attached to a tree at chest height at the location where the dam was originally captured. Exit holes were exposed after the nest box was firmly attached to the tree so that individuals could exit freely.

Wild juvenile deer mice were also sampled in this experiment. Wild deer mice were identified as juveniles based on their weight (<14g) and their smoky grey pelage. Based on these attributes individuals are expected to be approximately three-to-five weeks of age (Banfield 1974). Juvenile deer mice captured in the wild were subjected to the same sampling protocol as captive born individuals. A subset of wild caught juvenile deer mice were then translocated into captivity, while the remaining deer mice remained in the wild. Wild caught deer mice were then sampled again after two-weeks. This experimental design would allow translocation associated changes in GMCs α -diversity to be related with changes in host immunocompetence.

2.3.2 DNA extractions and bioinformatics

Methodology involved for microbial DNA extractions, PCR, amplicon sequencing, and bioinformatics from collected fecal samples are identical to those conducted in *Chapter 1* (see sections 1.3.4 – 1.3.7).

2.3.3 α -diversity

Methodology involved for measuring α -diversity for GMCs are identical to those in *Chapter 1* (see section 1.3.8).

2.3.4 Hematocrit ratios and blood smears

Blood samples (~80-100 μ L of whole blood) were collected via tail snips in 100 μ L Microvette tubes (Sarstedt Inc., Montreal, QC, Canada). Part of the blood samples collected were used to determine hematocrit (packed red-blood cell to plasma ratio). 10 μ L of Blood was collected in capillary tubes and centrifuged at 3,000 RPM for 5 min to separate blood cells from plasma.

The proportion of red blood cells and plasma within each sample was then measured (performed in duplicates). Hematocrit has been previously shown to display a non-linear relationship with fitness proxies (i.e. recruitment and longevity) in house wrens (Bowers et al. 2014), therefore residuals were used to generate a linear relationship, using the median as the optimal level, rather than the mean due to the data being right skewed.

For each sample two blood smear slides were created to assess differential white blood cell counts (DWBC) as well as the number of white blood cells (WBC) per 10,000 red blood cells. Slides were set in the field using methyl alcohol (1-2 s), then stained using Wright's Staining method (KwikTM –Diff stain kit, Thermo Electron Corporation), following manufacturers instructions. Total number of WBC and neutrophil:lymphocyte were used as proxies for basal immune investment (Tian et al. 2015). Differential WBC counts were performed by counting 100 leukocytes, recording the number of lymphocytes, neutrophils, monocytes, basophils and eosinophils. WBC counts were performed by counting the number of white blood cells identified per 10,000 RBCs counted.

2.3.5 Bacteria killing assay

The remaining blood sample (~70 μ L of whole blood) was centrifuged at 30,000 rpm for 5 min to separate the plasma from packed red-blood cells. The plasma was then extracted and stored in a 1.5 mL Eppendorf tube at -20°C for ~3-8 weeks (depending on when the mouse was samples), before being transferred to a -80°C freezer for ~4 months.

Bacteria killing assays measure an individual's ability to eliminate potentially harmful bacteria *ex vivo*. *E. coli* strain ATCC#8739 (also used by Martin et al. 2007, French and Neuman-Lee 2012, Schneeberger et al. 2013, Brown and Shine 2014) was used for the bacteria killing assay. This strain of *E. coli* is targeted by the complementary branch of the innate immune system is

responsible for eliminating harmful microorganisms by stimulating inflammatory responses as well as attacking foreign cells membrane structures (i.e. lysis) and mediating phagocytosis (Demas et al. 2011).

Agar plates were made using 40 g of agar per 1 L of distilled water and then plated on petri dishes (60 mm x 15 mm). Methodology for the bacteria killing assay used in this experiment followed instructions outlined by Millet *et al* (2007), using *E.coli* (ATCC #8739). To conduct the bacteria killing assay 5 μ L of plasma was added to a mixture of 95 μ L of CO₂-independent media (Gibco and Invitrogen; Life technologies, Grand Island, NY, USA) and 200 mM L-glutamine. The CO₂-independent media/L-Glutamine mixture, was made by combining 29.4 ml of CO₂-independent media and 0.6 ml of L-Glutamine. *E. coli* bacterial colonies were grown in an autoclaved test tube for ~24 hrs in an agar nutrient broth which was incubated at 37°C. The *E.coli*/nutrient broth solution was then vortexed, afterward the concentration of the solution was determined by count using a hemocytometer. Once the initial concentration was determined, the solution was diluted to obtain a final concentration of 50,000 cells/mL. 20 μ L of the diluted *E.coli* solution [50,000 cells/mL] was then added to the plasma/CO₂-independent media/L-Glutamine mixture in an autoclaved 1.5 ml Eppendorf tube, vortexed, and incubated at 37 °C for 30 minutes. After incubation, samples were vortexed and then 40 μ l (determined by preliminary tests) was plated and spread on agar plates, using a sterile swab. Each sample was then plated in duplicate, including control and negative control plates. Negative control plates containing only the CO₂-independent media/L-Glutamine mixture were also prepared to ensure that no contamination had occurred. Plates were stored upside down and incubated for 24 hours at 37 °C. Bacterial colonies were then counted and compared to the control plates which did not contain any plasma. Bacteria killing ability (BKA) was calculated as: 1-(average number of bacterial colonies formed on sample

plate/average number of bacterial colonies formed on control plates). The mean of replicate plates was calculated to obtain an individual's final BKA score.

2.3.6 Statistical analysis

Chao1 and Fisher's α -diversity indices both produced normal data, therefore parametric tests were used. Non-parametric data analysis was used when examining Shannon's diversity due to the non-normality of the data.

Based on the data's normality ANOVA and Kruskal-Wallis tests were used to examine between group differences of alpha diversity index scores during the initial and post-translocation capture periods. Tukey's multiple comparisons of means or Dunn's test were then conducted to identify significant differences between groups. Two-way ANOVAs were used to determine significant changes within groups between initial and post-translocation experimental periods.

Feature scaling normalization method was applied to BKA assay results to account for the differences in variance between sample runs, allowing them to be comparable. A disadvantage of the feature scaling normalization technique is that it does not represent true BKA scores, however, it allows for comparisons among individuals. A non-parametric multiple kernel linear regression model was used to identify relationships with functional immunity measurements (i.e. BKA) and α -diversity. Count measurements (hematocrit residuals, neutrophil:lymphocyte [N/L], and number of total number of WBCs) were also assessed using a non-parametric multiple kernel linear regression. An unpaired Mann-Whitney U test was used to assess the difference in BKA between captive-born and wild-born individuals, using samples from the initial capture experimental phase. Spearman's correlation test was used to determine if there was a significant relationship between the number of days samples were stored and BKA, a necessary test when plasma samples are stored for an extended period of time (Schneeberger et al. 2013; Jacobs and Fair 2016).

2.4 Results

In total 47 different deer mice had their fecal and blood samples collected, 17 of those individuals had both fecal and blood samples collected during twice, two weeks apart to assess how changes in GMCs correlate to changes in immunocompetence measurements. Therefore in total 64 fecal and blood samples were obtained. Not all individuals could be re-captured, therefore they could not all be resampled.

2.4.1 White blood cell differentials and hematocrit ratios

N/L ratios and total WBCs were marginally correlated with gut microbiome community (GMC) α -diversity levels when using Chao1 (N/L: $p = 0.078$; hematocrit: $p = 0.99$; WBC counts: $p = 0.093$) and Fisher's (N/L: $p = 0.070$; hematocrit: $p = 0.96$; WBC counts: $p = 0.093$). However, correlations only explained 15% and 17% variation, when using Chao1 and Fisher's indices respectively. Hematocrit ratio residuals showed no significant correlation with any of the alpha diversity indices. No correlations were present between alpha diversity and immune measurements when using Shannon's diversity index.

2.4.2 Bacteria killing assay

Bacteria killing ability (BKA) was positively correlated with the date of sample collection (Spearman correlation, $\rho = 0.39$, $p < 0.01$, $n = 62$). Length of storage was subsequently used as covariate when analysing BKA results. Overall BKA did not show any significant relationship with GMC α -diversity (Chao1: $t_{1,62} = 1.00$, $R^2 = 0.07$, $p = 0.32$; Fisher: $t_{1,62} = 1.13$, $R^2 = 0.04$, $p = 0.26$; Shannon: $t_{1,62} = 0.705$, $R^2 = 0.06$, $p = 0.48$; **Figure 2-1**). BKA scores ranged from 7-99% bacteria killing ability.

When grouped by place of birth (initial samples only) there was a marginal difference in BKA ($W = 372$, $p = 0.053$, **Figure 2-2**) between captive and wild born juvenile deer mice,

indicating there may be some environmental influence on BKA, however, it does not appear to be directly related to α -diversity of an individual's gut microbiome community. Additionally, when using Chao1 and Fisher's α -diversity indices there was no significant correlation between the change (post-translocation - initial sampling period) in gut microbiome α -diversity and in BKA between sampling periods (Chao1: $t_{2,15} = 1.29$, $R^2 = 0.01$, $p = 0.21$; Fisher: $t_{2,15} = 1.22$, $R^2 = 0.03$, $p = 0.24$). However, a marginal relationship was seen when using Shannon's diversity index to examine the relationship between the change in GMC and bacteria killing ability between capture periods (Shannon: $t_{2,15} = 2.13$, $R^2 = 0.13$, $p = 0.05$, **Figure 2-3**).

Storage time for plasma samples may have had an effect on BKA results, with the storage time in this study ranging from 0.5-2 months at -20°C followed by a 7 month period at -80°C , however, previous studies examining bacterial killing ability of deer mice, have used frozen plasma before when using assessing BKA using the plating method (opposed to spectrometry method). Samples used in a study by Martin et al. (2007) stored samples for 1.5 months at -80°C before analysis, as well an additional study on neotropical bats had samples stored for 81 days (Schneeberg et al. 2013).

2.4.3 Microbe-immune relationships

Linear regressions were used to test for significant correlations between operational taxonomic units (OTUs) whose relative abundance in gut microbiome communities was linked to the either captive or natural environments (*See Chapter 1*). A *Bacteroidetes* spp., (OTU 18: $t_{1,62} = -2.36$, $R^2 = 0.018$, $p = 0.088$), showed a negative correlation with BKA, while another *Bacteroidetes* spp. (OTU 9: $t_{1,62} = -1.779$, $R^2 = 0.020$, $p = 0.078$) and an *Oscillospira* spp., (OTU 185: $t_{1,62} = 2.174$, $R^2 = 0.032$, $p < 0.05$) showed a positive correlation with individual's BKA.

2.5 Discussion

2.5.1 α -diversity and immunity

BKA was marginally increased in wild individuals compared to captive individuals, however it, did not show any correlation with gut microbiome α -diversity. When using Shannon's diversity index however, a marginally significant relationship between the change in GMC α -diversity and BKA score between capture periods. This result provides some evidence for a relationship between GMCs and host immunocompetence, however the relationship only explain 13% of the variance. These results complement previous findings that wild mice tend to have better immune responses than captive mice (Abolins et al. 2011), however, failed to provide evidence for a strong correlation between immune system components and gut microbiome diversity (Mulder et al. 2009, 2011). Exogenous factors including, parasite load, food availability, and stress may have a larger impact on host immunocompetence than gut microbiome α -diversity. A Previous study examining gene expression and pathways in piglets placed in indoor and outdoor environments, found that individuals expose to outdoor environments had increased microbial exposure as well as expression of immune related genes (Mulder et al. 2011). Mulder et al's., (2011) methods were similar to ours, using early life stage individuals (piglets) placed in different environments, however, when measuring immune responses focus was placed on gene expression and pathways rather than functional eco-immunological methods. Eco-immunological approaches to assess immunocompetence may reveal more information regarding host fitness than other methods but, are inherently harder to conduct, especially when working with wild populations in the field rather than with laboratory populations.

Both neutrophil:lymphocyte ratios and WBC counts had weak relationships with gut microbiome α -diversity. Future research is needed before the connection between gut microbiome diversity and host immunocompetence can be fully understood in wild populations.

Gut microbiome components have been associated with immune system upregulation (Round and Mazmanian 2009, Mulder et al. 2011, Thaiss et al. 2016), which may be more frequently activated under increased α -diversity levels (Stappenbeck et al 2002). However, there are a number of other factors that individuals in the wild may have been exposed to, that could have increased immune investment, such as parasitism, which is not present in captive environments. Further investigation is necessary to determine whether increased neutrophil:lymphocyte and total number of WBC is activated through increased gut microbiome diversity or is an artifact of exposure to other environment factors such as parasites.

2.5.2 Microbe-immune relationships

Three OTUs were associated with functional innate immune responses, however, no clear pattern was observed. *Bacteroidetes* spp., showed mixed correlations with BKA, containing OTUs which either showed a positive or negative correlation with BKA. Due to the lack of information pertaining to these OTUs, classified as *S24-7* family, it is difficult to expand on their potential effect on host health, emphasizing the need for increased studies on this group of bacteria. An *Oscillospira* spp., (OTU 185) was also correlated with increased BKA. Previous research has also demonstrated that *Oscillospira* spp., are commonly found within mice GMCs and may be to thrive when individuals are placed in natural environments (Baxter et al. 2015, *see* Chapter 1). These relationship suggest that the presence of environment-microbe-host interactions could be important to consider when dealing with captive populations.

2.5.3 Conservation perspectives

Recent research has suggested that changes in GMCs may be associated with changes in host fitness, with particular emphasis being placed on species involved in reintroduction conservation projects (Redford et al. 2012). This study attempted to decipher environment-

microbe-host interactions to determine how these interactions may influence host fitness among captive populations. No clear relationship was seen between GMC α -diversity levels and immunocompetence in deer mice. Although, a weak relationship was seen when using Shannon's diversity index to examine changes in BKA and GMC α -diversity, and wild deer mice were marginally better at BKA, providing some evidence that GMCs may affect individual's immunocompetence in wild populations. However, further research is need to determine the magnitude of this impact. One explanation as to why this study only found a weak relationship is that α -diversity may not be as important in maintaining increased immunocompetence levels as certain microbes or groups of microbes (Chung et al. 2012). Alternatively, health benefits provided by a diverse gut microbiome may be over shadowed by other factors, which may influence host health including: food availability, parasite load, stress etc. Future research should continue to assess immunity associated responses in relationship to GMCs using natural populations, to benefit captive-based conservation programs.

2.5.4 Conclusion

This study is one of the first studies to examine correlations between GMCs and immunocompetence, which could reveal environment-microbe-host interactions with potential fitness related consequences for host species. Numerous studies have demonstrated microbiome differences between captive and wild populations (Villers et al. 2008, Xenoulis et al. 2010, Nakamura et al. 2011, Cheng et al. 2015, see *Chapter I*), however, few have attempted to associate differences in gut microbiome communities with potential fitness consequences (Mulder et al. 2009, 2011). This study used an eco-immunological approach and provides some evidence of how GMCs may influence immunocompetence as well as an introductory example for future research attempting to determine fitness consequences associated with gut microbiome diversity in wild

populations. Future studies should continue to move beyond simply identifying differences between gut microbiome communities and attempt to associate gut microbiome characteristics with host fitness, providing a beneficial tool for captive-based conservation programs.

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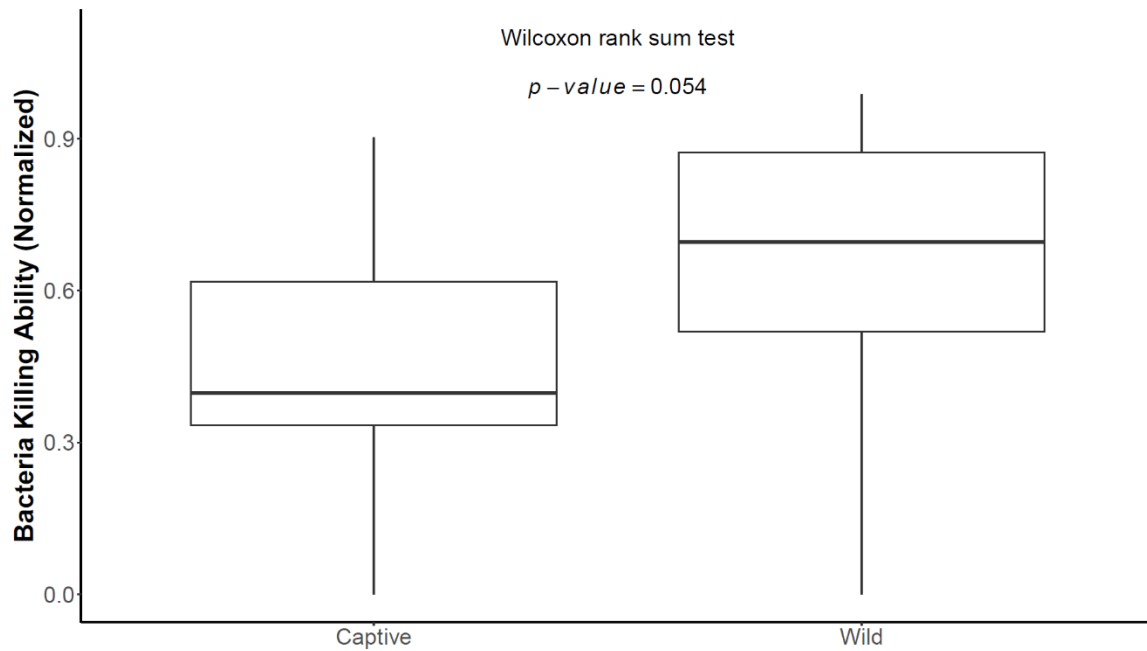


Figure 2-1: Mann-Whitney U test was used to test for significant differences between bacteria killing ability of individuals born in captivity ($n = 27$) and the wild ($n = 24$). A marginal difference was seen between the two groups ($W = 372$, $p = 0.053$).

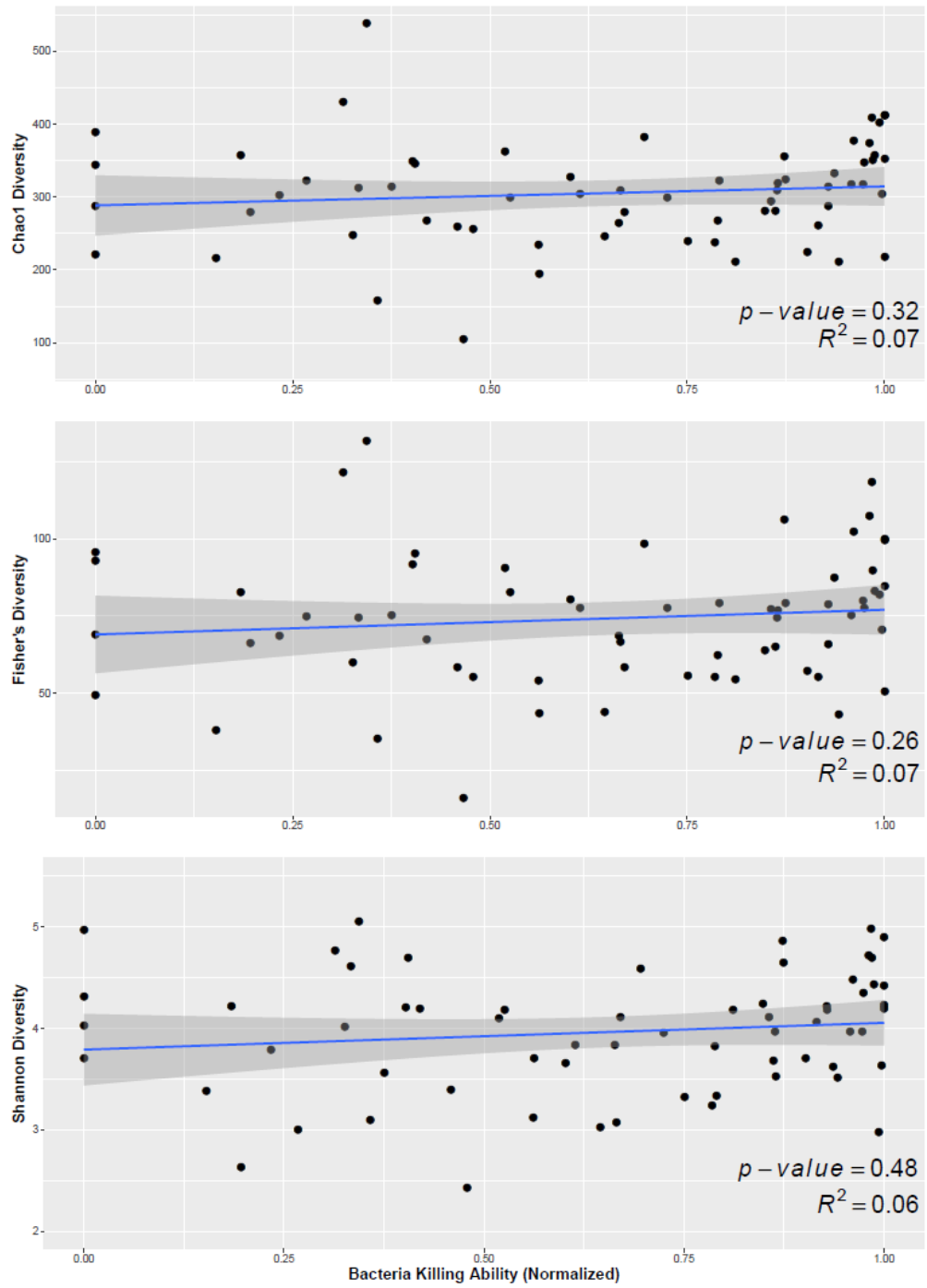


Figure 2-2: No significant correlations appear to be present between Chao1, Fisher's, or Shannon's α -diversity indices and bacteria killing ability (n = 63).

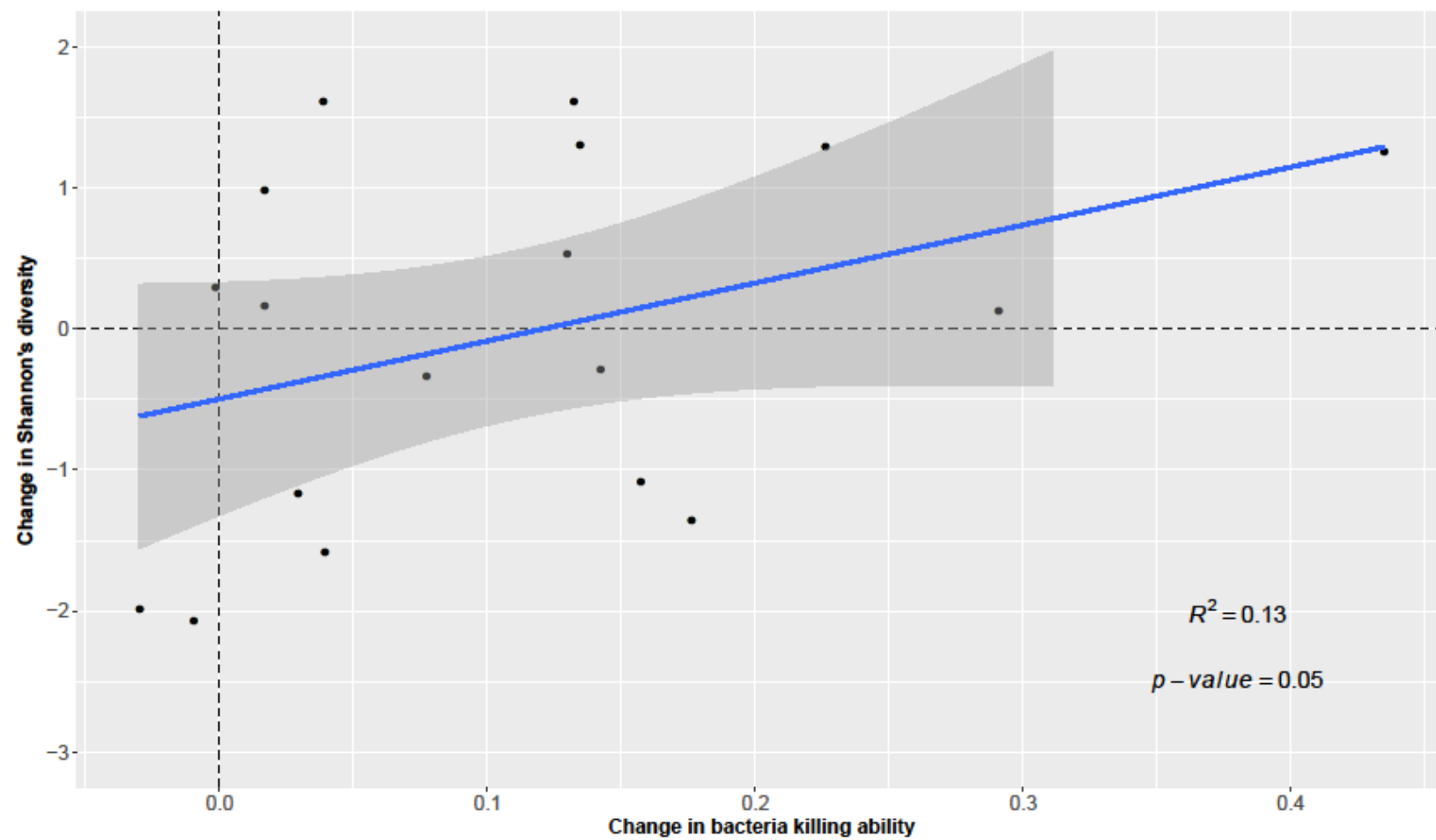


Figure 2-3: A marginally significant correlation ($R^2 = 0.13$, $p = 0.05$) was seen when comparing the change in Shannon's diversity and bacteria killing ability over the two week translocation period ($n = 17$).

General Discussion

Every environment on earth harbours a diverse array of microorganisms, some of which have developed strong relationships with vertebrate hosts. Microorganisms inhabiting the gastrointestinal tract of vertebrates have been shown to benefit the physiological development and health of their hosts (Round and Mazmanian 2009, Lee and Mazmanian 2010, Shapiro et al. 2014, Thaiss et al. 2016). Microbes that colonize and become established within the gastrointestinal tract of vertebrates form unique communities (Ley et al. 2008a), however, the factors controlling the formation of these communities are poorly understood, especially in natural populations. Limited research has been conducted on gut microbiome communities (GMCs) of wild populations (Hird 2017), with most current research being performed on laboratory mice. An increased understanding of how gut microbiome communities form under natural conditions may provide meaningful information regarding environment-microbe-host interactions, which may influence host health and overall fitness, providing useful knowledge towards the conservation of vertebrate hosts.

The aim of this thesis was to analyze how gut microbiome communities differentiate between captive and wild environments, as well as track changes that occur during translocations. It was hypothesized that gut microbiome communities would be influenced by exposure to the exogenous environment. Subsequent predictions stated that GMCs would display enterotypes based on an individual's surrounding environment. GMCs (α -diversity and composition) of deer mice translocated to different environments were expected to homogenize with deer mice that inhabit the same environment. Maternal effects were hypothesized to occur during the initial capture phase, with dam GMCs being distinctively more similar to their own offspring compared to other litters. Maternal influences however were expected to disappear post-translocation due to

extrinsic environmental influences masking maternal influences. Deer mice born in captivity were expected to have more similar GMCs to their siblings rather than counterparts from other litters or wild-caught individuals, however post-translocation unique enterotypes were not expected to exist within litters. The second objective of this thesis was to examine how gut microbiome α -diversity influences host immunocompetence. Gut microbiome α -diversity was hypothesized to be correlated with an individual's immunocompetence ability. Mice born in the wild and possessing higher levels of α -diversity were predicted to possess optimal hematocrit ratios, increased white blood cell and neutrophil:lymphocyte counts, as well as higher BKA compared to mice born in captivity whose gut microbiome communities are less diverse.

Gut microbiome communities between captive and wild environments – α -diversity

Evidence was found supporting the hypothesis that exposure to the exogenous environment heavily influences GMCs. Individuals in captivity were associated with lower gut microbiome α -diversity compared to individuals in the natural environment. Additionally individuals translocated from captivity to the wild experienced an increase in α -diversity, while individuals reciprocally translocated experienced a decrease. The reciprocal relationship between the translocation groups suggest that continued exposure to a diverse source of microorganisms is necessary to maintain a diverse gut microbiome community. Upon switching to a solid food diet, deer mice held in captivity experienced an increase in GMC α -diversity, however, the increase was not proportionate to the increase seen in deer mice translocated from captivity to the wild. These findings provide evidence complementary to Kohl et al. (2014), that animals in captivity may not be able to obtain a GMC that is as diverse as GMCs found in wild animals. However, this study also provided evidence that upon being translocated into the wild from captivity, individuals may be able to acquire a highly diverse GMC over time. Continued exposure to the exogenous environment is

also important to maintaining diverse GMCs and should be considered when translocated animals from wild to captive environments.

Results from this experiment complement previous studies that have examined microbial communities between captive and wild individuals (Villers et al. 2008, Xenoulis et al. 2010, Kohl et al. 2014, Cheng et al. 2015), however, it is also important to mention that conflicting results have also been demonstrated (Mulder et al. 2009, Kohl et al. 2017). Conflicting results are paradoxical since natural environments contain a more expansive pool of microbes which may contribute to an individual's GMC compared to captive environments. Within natural environments microbes may be acquired from more diverse diets (Kohl et al. 2014), host richness (Gavish et al. 2014) and ranges (more exposure to different biogeographical factors [i.e. soil, water etc.]) (Lee et al. 2010, Lankau et al. 2012, Linnenbrink et al. 2013). Results from Mulder et al.'s., (2009) study, which found that outdoor reared piglets had lower GMC α -diversity levels compared to indoor reared piglets, are particularly surprising since their experimental design contained a number of similarities as this present study, yet demonstrated conflicting results. Conflicting results within the literature suggest the need for more studies focusing on differences between microbial communities in wild and captive populations, for an increased understanding of factors influencing GMC α -diversity.

Gut microbiome communities between captive and wild environments – β -diversity

Complementary to results pertaining to α -diversity, analysis on β -diversity provided further evidence supporting the hypothesis that environmental exposure influences GMCs. During the initial sampling stage the GMCs of deer mice in captivity and wild environments clustered based on environment. When captive born deer mice were translocated for two-weeks into the wild, the

GMCs of translocated individuals clustered closer to neighbouring deer mice in the wild, opposed to their siblings that remained in captivity.

At the phyla level all mice were dominated by Firmicutes and Bacteroidetes, a common trend among mammals (Ley et al. 2008a), however mice in captivity contained a higher relative abundance of Bacteroidetes than Firmicutes, whereas wild mice displayed the opposite. Trends were seen regarding specific microbial families and exposure to natural environments. *Ruminococcaceae*, *Helicobacteraceae*, and *Lachnospiraceae* were all shown to have an increased abundance in the GMC of deer mice in natural environments. OTUs belonging to these three families were identified as being enterotype-defining for individuals in natural environments, and showed reciprocal changes between translocated mice.

Ruminococcaceae and *Lachnospiraceae* (both Firmicutes) have been associated with plant based diets, suggesting that their dominance in wild individuals, may be caused by food sources not accessible in captive environments (Mackie et al. 2003, Flint et al. 2008, Biddle et al. 2013). *Helicobacteraceae* were also connected with exposure to the natural environment and despite being associated with being pathogenic, are expected to display commensal relationships within murine hosts (Baxter et al. 2015). Microbial species belonging to these families were shown to be deterministic of the wild enterotype. Captive animals may be more likely to obtain GMCs resembling those of wild animals, through exposure to these microbes. However, our understanding of these microbes and their influence on their hosts are poorly understood and should be further studied to determine the potential benefits they may provide their hosts. Future research should also further investigate relationships between hosts and *Helicobacteraceae* spp., since they are typically opportunistic pathogens, yet maintain a noticeable presence in the GMC of wild animals. If future studies determine that the diversity of GMCs in wild animals provide

fitness benefits then the microbial families associated with the wild enterotype in this study may provide a starting point when determining which microbes may be responsible for providing potential fitness benefits.

Evidence from this study supported the hypothesis that maternal influences only display short-term effects and are quickly masked upon exposure to natural environments. Although previous studies have provided evidence of maternal influences in controlled laboratory studies (Bension et al 2010, Funkhouser and Bordenstein 2013), this study suggests that in the wild these influences are quickly masked by more influential factors such as exposure to the exogenous environment. Differences in the GMCs of male and female deer mice captured in the wild were present in wild individuals, however, they only explained ~5% of the total GMC variation. A lack of difference between the GMC of male and female deer mice in captivity, may have been caused by the influence of co-housing, which has been shown to make GMCs more similar between individuals. Admittedly this study used juveniles which had not experienced differential physiological changes associated with different sexes, possibly explaining why sex differences explained such little variation.

Environment-microbe-host interactions

Environment-microbe-host interactions have received little attention in the literature, with most studies focusing either on environment-microbe or microbe-host relationships. This is one of the first studies to examine how microbes associated with the natural environment influences host health. Although wild mice were found to have marginally increased bacteria killing ability (BKA), no correlation was found directly with GMC α -diversity. However, a weak correlation was found between the change in GMC and immunocompetence, suggesting that large changes in GMCs may have potential fitness consequences. Further research should be conducted to

determine the magnitude of impact GMCs can have on their hosts in terms of fitness, to aid in the understanding of how a lack of microbial exposure in captivity may infer fitness costs. Marginal correlations were found between GMC α -diversity and proxies for basal immune investment, however, further studies will be need to clarify results.

Some OTUs were associated with different immune measurements, however, no clear phylogenetic pattern was observed. While limited understanding of how exactly these OTUs influence host health can be explain by this present study, future studies should consider examining the affect health-related OTUs found in this study. Determining the role of different microbes within GMCs using wild populations would be difficult. An increased understanding of how particular OTUs impact their hosts would benefit from using laboratory-based studies, where specific microbe-immune interactions are more easily overserved.

Conservation perspectives

Recent research has shown that GMCs may be important factors to consider in species involved in captive based conservation programs (Redford et al. 2012). Regarding applicability to captive based conservation programs, results from this study have revealed that upon reintroduction to natural environments, juveniles may be able to have their GMCs rapidly change in terms of both α - and β -diversity. Within this study, deer mice in the wild possessed increased abundances of *Ruminococcaceae*, *Helicobacteraceae* and *Lachnospiraceae* spp., suggesting that these families thrive when individuals are placed in natural environments compared to captivity. Increased exposure to these microbial families when in captivity may provide captive animals with GMCs more similar to those of wild animals. Although no clear implications towards host health or fitness were revealed in this study, future research is encouraged to continue to investigate environment-microbe-host relationships within wild populations using eco-immunological

approaches, in an attempt to provide captivity-based conservation programs with essential knowledge.

Future directions

Future research should use this present study as an introductory example how to conduct further studies attempting to go beyond simple microbiome description and start to focus on environment-microbe-host interactions as well as how GMCs may influence host fitness. Previous studies have looked at how captivity affects gut microbiome communities, however no previous studies have looked at how GMCs are influenced when individuals are translocated from captivity to the wild. This study not only fills gaps within the existing literature but also provides a number of new and focused directions that future research can take (**Figure 3-1**). Different α - and β -diversity metrics should also be used in future studies to ensure comparability between experiments. Additionally, future studies should sample individuals at multiple time points throughout experiments, to make sure they are not simply catching a one-time ‘snap-shot’ of a GMC (Maurice et al. 2015, Bobbie et al. 2017). As shown in this current study, as well as in previous research (Maurice et al. 2015, Bobbie et al. 2017) GMCs can change rapidly over time, therefore repeated sampling should be considered in all future experiments.

Studies examining GMCs should continue to examine which specific groups of microbes are driving the changes between GMCs in different environments. Understanding which microbes dominate GMC may reveal important environment-host-microbe interactions; interactions which may be important in vertebrate conservation efforts. Increased future efforts should focus on investigating the potential fitness implications of increased microbial diversity, as well as particular microbes’ in an attempt to provide meaningful guidance for captivity based conservation programs.

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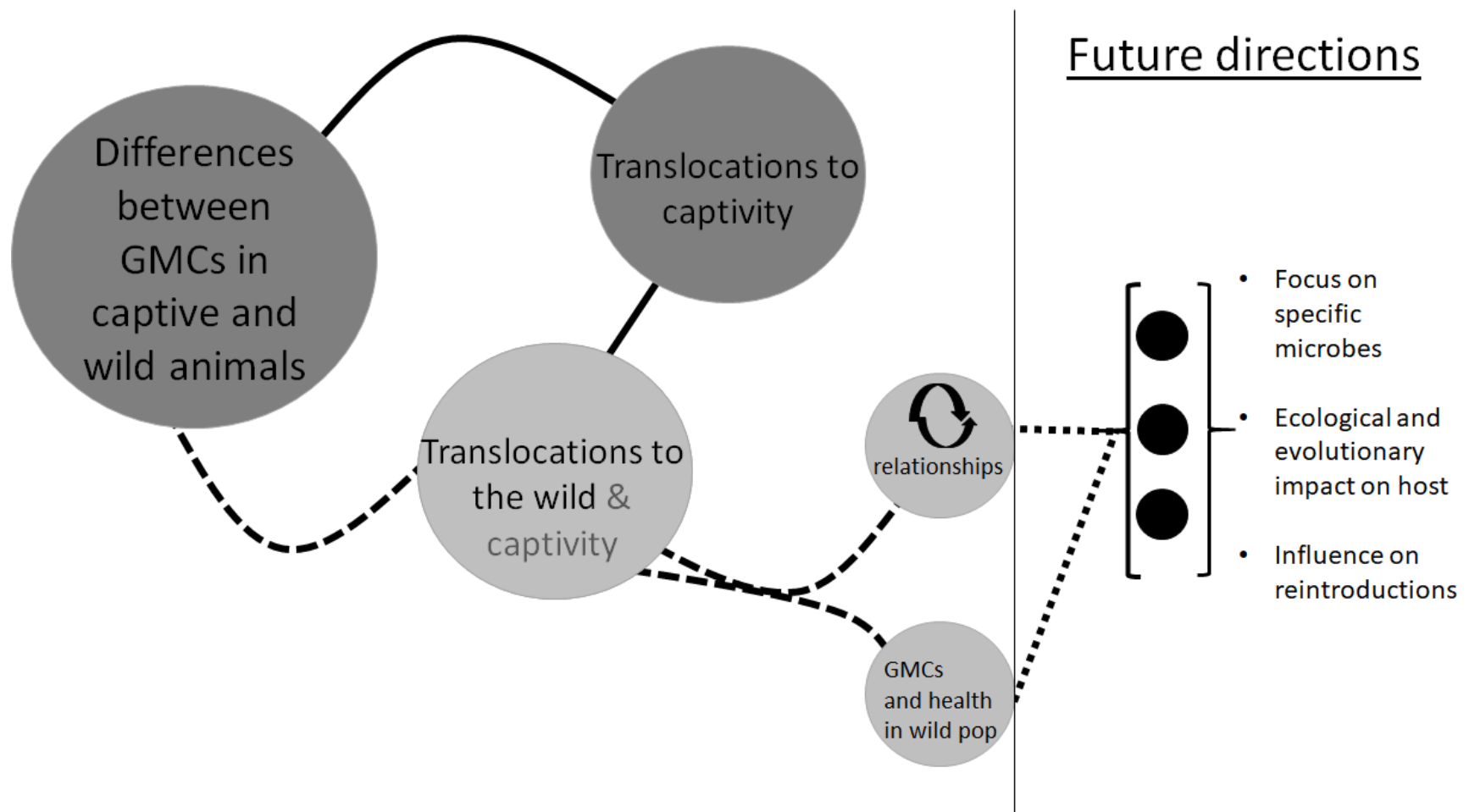


Figure 3-1: A visual representation of the how this study fills current gaps within the existing literature and how the findings act as a starting point from which future research can start from. Dark grey circles represents the focus of existing research, while the light grey circles represent the focus of this study (research that current does not exist within the current literature). Future directions for research is also outlined.